

# CANCER RESEARCH

VOLUME 11

JULY 1951

NUMBER 7

## Effect of Pyridoxine and Desoxypyridoxine on Rat Fibrosarcoma Grafts\*

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Several reports in the literature on induced and transplanted tumors suggest that vitamin B<sub>6</sub> may be a factor of importance relative to incidence and growth of induced and transplanted tumors. When the Flexner-Jobling carcinoma was implanted in rats partially deficient in pyridoxine, the percentage of takes was lower (66 versus 85 per cent), the number of regressions higher (30 per cent versus none), and the size of the tumors smaller than in control animals (20) on an equivalent caloric intake. Similar results were reported for mice inoculated with a fibroid sarcoma (56 versus 76 per cent takes) or the Yale carcinoma No. 1 (71 versus 92 per cent takes). The incidence of skin tumors induced by methylcholanthrene painting likewise was lower in pyridoxine-deficient animals (36 versus 62 per cent). With diets containing *p*-dimethylaminoazobenzene the incidence of rat liver tumors paralleled the amount of available pyridoxine, so that when vitamin B<sub>6</sub> in the diet was increased or reduced, tumor incidence was correspondingly increased or reduced (25, 26). Benzpyrene-induced epithelial tumor incidence in mice chronically deficient in pyridoxine was 22 per cent lower than in controls, and this effect was independent of caloric intake, according to Boutwell and Rusch (4). A lowered incidence of benzpyrene-induced epithelial tumors in mice on low B-vitamin rations was observed by Boutwell, Brush, and Rusch (3) and interpreted to be the result of vitamin B<sub>6</sub> deficiency. Other workers (2) reported that if vitamin B<sub>6</sub> was omitted from an otherwise adequate mouse diet, there was a marked decrease in the growth rate of Sarcoma 180.

\*Supported in part by grants-in-aid from Mr. and Mrs. Lewis J. Moorman, Jr., and the American Cancer Society (#CBC5).

Received for publication August 7, 1950.

On the other hand, Morris (27, 28) noted that vitamin B<sub>6</sub> deficiency seemed not to affect growth of spontaneous mammary tumors in C3H mice. Nontumor-bearing B<sub>6</sub>-deficient mice were able to survive for 8 weeks, but they lost weight, developed necrotic tails and encrusted ears, lost hair, and became partially paralyzed. The tumor-bearing mice on the same deficient diet, however, exhibited no symptoms of this type, and tumor growth appeared unaffected. Pyridoxine did not stimulate growth of the Rous chicken sarcoma (23), although a positive response was obtained with folic acid, niacinamide, calcium pantothenate, riboflavin, and cholic acid.

Since Woods's (40) report on growth inhibition by metabolite analogs, other vitamin inhibitors have been used by a number of investigators (35, 41, 42) to demonstrate deficiency diseases in animals. This concept was recently discussed by Greenberg and Schulman (15) in relation to cancer research, and a number of reports have appeared on the use of certain metabolite analogs as tumor growth inhibitors. Stoerk (33) used the pyridoxine analog desoxypyridoxine for experiments on lymphosarcoma 6C3H-ED in hybrid mice (C strain females crossed with C3H males). His experimental animals were kept on a B<sub>6</sub>-deficient diet for 3 weeks, and 300 µg. of desoxypyridoxine was added per milliliter of drinking water. Pyridoxine supplemented the diet of control animals. Four weeks after implantation, twelve of fourteen control animals (86 per cent) had tumors, while only two of twelve (17 per cent) on the desoxypyridoxine diet had developed tumors. Tumors of animals on the B<sub>6</sub>-deficient desoxypyridoxine diet were much smaller than those of controls.

Other antagonists, e.g., isoriboflavin (35), folic acid analogs (5, 6, 10, 19, 32, 37, 38, and 39),

purine and pyrimidine analogs (6, 11, 13, 14, 18, 21, 31, and 36) have been used to produce or intensify the effects of certain deficiencies in attempts to control growth of tumors and leukemia.

Our investigations were carried out to determine whether pyridoxine might be a factor influencing implantation of the rat tumor used in this laboratory. A previous study (24) had shown that the percentage of takes could be correlated with age of the host. It was postulated that the phenomenon observed might be attributable to the nutritional status of the host at different ages. A rational approach to the solution of this problem, at least as far as vitamin B<sub>6</sub> was concerned, was possible through the use of supplementary pyridoxine and its antagonistic analog desoxypyridoxine.<sup>1</sup> The transplantable rat fibrosarcoma previously used was utilized in this study.

They were segregated into groups of 30 and treated during the next 2 weeks as indicated below. Subsequently all received implants of grafts from the same stock, and 20 days later takes were recorded as shown in Table 1. Each of thirty control animals received thirteen daily intraperitoneal injections of 1 ml. distilled water. Another group was given a series of thirteen pyridoxine injections of 100  $\mu$ g. each in 1 ml. distilled water. Total pyridoxine given per animal amounted to 1,300  $\mu$ g. Each animal of the third group received intraperitoneally 5 mg. of desoxypyridoxine dissolved in 1 ml. distilled water per day. The total amount given to each animal was 65 mg. As may be seen in Table 1, the highest percentage of takes was observed in the pyridoxine-treated rats.

For series 3 another group of 90 male rats, also 5 weeks old, was used. During a 2-week period, the

TABLE 1  
EFFECT OF PYRIDOXINE AND DESOXYPYRIDOXINE ON A TRANSPLANTED RAT FIBROSARCOMA

SERIES NO.	CONTROLS			PYRIDOXINE-TREATED			DESOXYPYRIDOXINE-TREATED		
	No. rats	No. takes	Per cent	No. rats	No. takes	Per cent	No. rats	No. takes	Per cent
1	27	7	26	28	10	36	28	2	7
2	25	4	16	27	13	48	24	3	12
3	29	2	7	26	9	35	29	4	14
Totals and averages:	81	13	16	81	32	40	81	9	11

## METHODS AND RESULTS

Ninety 5-week-old male rats were used in series 1. All had been raised on a Purina Laboratory Chow diet and had remained on this regimen during the course of the experiment. The animals were randomly segregated into three groups. Thirty rats of the control group each received eight 1-ml. intraperitoneal injections of distilled water during the next 14 days. Each of the 30 animals in the second group received eight intraperitoneal injections of 100  $\mu$ g. pyridoxine during the 2-week period. Animals of a third group were each given eight 1-ml. injections of distilled water containing 5 mg. of desoxypyridoxine per milliliter. After the 2-week treatment period, when the rats were 7 weeks old, implants from an 18-day stock tumor were made to all animals. Thirteen days later, the percentage of takes in each group was recorded (Table 1).

Ninety male animals comparable in age and weight to those of series 1 were used in series 2.

<sup>1</sup> Merck's pyridoxine (2-methyl-3-hydroxy-4,5-dihydroxy-methylpyridine) and desoxypyridoxine (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine hydrochloride) were used. The latter was furnished through the courtesy of Dr. Augustus Gibson.

30 control animals each received eleven injections of 1 ml. distilled water. Sixteen days after the first injection, and following the tumor implants, four more were administered. Each of another 30 rats received eleven intraperitoneal injections of 100  $\mu$ g. of pyridoxine before, and four more after, receiving tumor grafts, for a total of 1,500  $\mu$ g. per rat. The third group received an equal number of 10 mg. desoxypyridoxine injections, for a total of 150 mg. per rat. Tumors were palpated at 13 days, and the record is shown in Table 1.

Animals that received desoxypyridoxine gained only 40 per cent in weight, as compared to controls and pyridoxine-treated rats which gained 46 per cent. Of those that received desoxypyridoxine, only a small number developed minor cutaneous lesions on the tail, paws, snout, and ears, but none showed ventral curling of the distal portion of the tail.

Pyridoxine-treated animals exhibited a higher average percentage of takes (40 per cent) than either controls (16 per cent) or desoxypyridoxine-treated rats (11 per cent). The difference in the latter two figures is not considered significant.

Another experiment was carried out with hamsters to determine whether pyridoxine-deficiency

symptoms could be established in these animals by administering desoxypyridoxine while they were kept on a diet of Purina Laboratory Chow. Two age groups with twenty hamsters in each were used. One group was 5 weeks and the other 10 weeks old. Ten animals of each age group were used for controls and ten for test animals. The test animals received a total of 190 mg. of desoxypyridoxine, given in 5-mg. intraperitoneal injections either daily or at intervals of not more than 3 days, over a period of 59 days (= 2.3 mg. per day average). There were no significant weight differences in either the 5- or 10-week test animals, as compared to their controls at the end of the test period, nor were any of the characteristic symptoms of vitamin B<sub>6</sub> deficiency observed such as described by others (29, 30).

### DISCUSSION

Since supplementary pyridoxine resulted in a higher percentage of takes, it is of interest to consider the minimum vitamin B<sub>6</sub> requirement of the rat. Most estimates (7, 8, 22) agree that the amount needed daily is about 10  $\mu$ g. For the hamster 3  $\mu$ g. per day was sufficient to prevent the acrodynia-like dermatitis which characterizes vitamin B<sub>6</sub> deficiency (29). There is some evidence that rats on a Purina Laboratory Chow diet may be near a minimum B<sub>6</sub> subsistence level. According to Wileke<sup>2</sup> of the Ralston-Purina Co., approximately 2  $\mu$ g. of pyridoxine per day would be supplied from 15 gm. of laboratory chow. Hence, a considerable amount of the daily vitamin B<sub>6</sub> requirement appears to be obtained from other sources; possibly it is provided by the bacterial flora.

Even if this explanation were correct, the question still remains why desoxypyridoxine administered in quantities up to 10,000  $\mu$ g. per day for 15 days, as in series 3, did not cause deficiency symptoms in most of the rats. The ratio of desoxypyridoxine to pyridoxine over this period, on the basis of the daily subsistence requirement of B<sub>6</sub> was approximately 1,000:1. Emerson (9) was unable to demonstrate deficiency symptoms by omitting pyridoxine from a rat diet, although when desoxypyridoxine was added, typical acrodynia developed after 55 days if the ratio of analog to vitamin was 50:1 or greater. It is possible, of course, that the period of administration of desoxypyridoxine in our experiments was not long enough for deficiency symptoms to develop in more than a few cases. Or, possibly, the tumor-bearing animals differed from normal rats, as the experiments of Morris (27) suggest. We did not treat nontumor-bearing

rats with desoxypyridoxine, hence have no data bearing on this point. Another possible explanation for failure of deficiency symptoms to appear in many rats is that desoxypyridoxine may not have acted as an inhibitor to pyridoxal or pyridoxamine, the other forms of vitamin B<sub>6</sub>. Perhaps the action is more involved, for, not only does this vitamin function in normal metabolism (1), but, according to the report of Keresztesy *et al.* (17), it may act indirectly by counteracting inositol inhibition of Sarcoma 180. Inositol inhibition of this tumor was also counteracted by *p*-aminobenzoic acid and other compounds such as thiamin, niacinamide, and leukopterin.

While instances of success in controlling tumor development were cited in the introduction, it might be worth pointing out that certain other reports indicate failure to control tumor growth by the use of desoxypyridoxine. Gregoire (16), for example, reported that lymphosarcoma development in young rats was delayed by a vitamin B<sub>6</sub> deficiency induced by certain quantities of desoxypyridoxine, but use of larger doses gave inconclusive results. Gellhorn and Jones (12), in a report on six clinical cases of which three were leukemia and three lymphosarcoma, stated there was no evidence that restriction of pyridoxine in the diet, together with administration of desoxypyridoxine for periods up to 2 weeks, had any therapeutic effect on lymphosarcoma or acute leukemia.

### SUMMARY

The incidence of fibrosarcomas, following subcutaneous grafting into 7-week-old control rats living on a Purina Laboratory Chow diet, was compared to that in rats which received supplements of pyridoxine and in others that were given desoxypyridoxine; 243 animals were used.

Pyridoxine supplements per rat totaled as much as 1,500  $\mu$ g. over a 2-week period before implantation and up to 400  $\mu$ g. following grafting. Desoxypyridoxine injections totaled as much as 110 mg. per rat preceding tumor injections and up to 40 mg. subsequently.

Tumor takes after 13–20 days in pyridoxine-treated rats averaged 40 per cent, as compared to takes of 16 and 11 per cent, respectively, in controls and desoxypyridoxine-treated animals. Pyridoxine deficiency symptoms were noted in only a few rats bearing tumors in the desoxypyridoxine-treated group.

Although this analog was also administered in relatively large amounts to nontumor-bearing hamsters on an identical basic diet over a period of 59 days, no vitamin B<sub>6</sub> deficiency symptoms were observed.

<sup>2</sup>H. L. Wileke, personal communication, 1949.

## ACKNOWLEDGMENT

The writer gratefully acknowledges the technical assistance of Norbert G. Gilles.

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# Nuclei from Normal and Leukemic Mouse Spleen

## II. The Nucleic Acid Content of Normal and Leukemic Nuclei\*

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In an earlier study of the distribution of pentosenucleic acid (PNA) among the various fractions derived from normal and leukemic spleen homogenates by differential centrifugation, it was found that the PNA concentration was greatly increased in a crude, unwashed "nuclear fraction" of leukemic spleen (11). It therefore seemed desirable to determine the nucleic acid content of uncontaminated nuclei. Since in leukemia the PNA is also elevated in the cytoplasm (11), a method for the separation of nuclei in a neutral medium in which contamination with cytoplasmic nucleoprotein should be minimal was developed. The isolation and analysis of nuclei from normal spleen have been reported in the first paper of this series (15). In this paper data on nuclei from spontaneous and transplanted leukemia are presented and compared.

The nuclei prepared from the spleens of mice with transplanted leukemia contain over 4 times as much PNA (per nucleus) as those from normal spleen; their desoxyribonucleic acid (DNA) and nitrogen contents are also greatly increased. In spontaneous leukemia, on the other hand, the nuclear PNA is but slightly increased, and the DNA and nitrogen contents are normal.

### MATERIALS AND METHODS

Mice of the Akm strain (2) were used. The normal mice and those used in the experiments on transplanted leukemia were 2-3 months old. The line of transplanted lymphatic leukemia, 9421, was the same as that used in previous studies (11, 12). Leukemic spleen, minced in saline and injected intraperitoneally, produced advanced leukemia in about 10 days. The spleen weights then averaged about 400 mg., an increase of about 200 per cent over the normal values. The mice with spontaneous leukemia were about 10 months old. Their

spleens were very large, weighing from 400 to 1,800 mg.

The nuclei were isolated by a procedure similar to that used on normal spleen (15), except that an increased amount of calcium chloride was added to the 0.88 M sucrose medium. With normal spleen, a concentration of 0.0018 M calcium chloride is sufficient to prevent stickiness and clumping of the nuclei, without causing any aggregation of the cytoplasm. In both spontaneous and transplanted leukemia, however, it was found necessary to increase the amount of calcium chloride to 0.0023 M to obtain the same effect. Staining techniques, nuclear counts, and nucleic acid and nitrogen analyses were carried out by the same techniques that were used for normal spleen (15).

Control experiments on the effect of calcium chloride were made as follows: One gm. of normal spleen was homogenized for 2½ minutes in a Potter-Elvehjem homogenizer with 9 cc. of 0.88 M sucrose (11). Four cc. of homogenate was then measured into a clean ground glass homogenizer. Two cc. of sucrose was added, and homogenization was continued for ½ minute. The homogenate was then transferred to a 15-cc. centrifuge tube. A second 4-cc. sample of the first homogenate was treated in the same way, except that 2 cc. of 0.88 M sucrose containing 0.0054 M calcium chloride was added, to give a final calcium chloride concentration of 0.0018 M. Both tubes were centrifuged at 1,000 g for 15 minutes. The volumes of the nuclear and cytoplasmic fractions were recorded and the cytoplasm suspension removed for nucleic acid analysis.

A similar experiment was carried out on transplanted leukemia spleen. Here the calcium chloride concentration of the 2 cc. of added sucrose was 0.0069 M, so that the final concentration of calcium chloride would be 0.0023 M.

### RESULTS AND DISCUSSION

The isolated nuclei appeared round, unclumped and undistorted, and resembled morphologically those seen in stained sections of leukemic mouse spleen. The large nucleoli showed up clearly.

\*The authors wish to acknowledge the assistance of the National Cancer Institute, Public Health Service, the Office of Naval Research, contract N6-ori-99, Task Order 1, the Atomic Energy Commission, contract AT(30-1)-910, the Barker Welfare Foundation, and the James Foundation of New York, Inc.

Received for publication January 8, 1951.

The results of the nitrogen, DNA, and PNA analyses are shown in Chart 1. All three are significantly increased in transplanted leukemia, while in spontaneous leukemia only a slight increase in PNA is found.

While the yields of nuclei were low (10–20 per cent) (15), the isolation procedure was the same for normal and leukemic spleen, except for the  $\text{CaCl}_2$  concentration. It is therefore probable that about the same proportion of cells was lost during filtration, either because the cells were larger or because they were imbedded in fibrous connective tissue, and that the same proportion of smaller nuclei was lost in the supernatants during the sedimentation and washing steps. The close agreement in nitrogen values between normal and spontaneous leukemic nuclei supports this explanation.

The use of calcium chloride raises the question of whether cytoplasmic PNA has been precipi-

tated by the calcium, since this might lead to contamination of the nuclei. Schneider (16) has found that calcium concentrations similar to those used here precipitate significant amounts of cytoplasmic PNA from liver homogenized in distilled water. Although no agglutination of cytoplasm or nucleoprotein precipitate such as that found in the liver homogenates (16) was ever observed, it seemed advisable to check the effect of calcium chloride by chemical analysis. The results of these control experiments (Table 1) are difficult to interpret. While the concentration of PNA in the cytoplasm is definitely lower when calcium has been added, the total volume of cytoplasm is significantly increased. As a result, the total amount of PNA left in the cytoplasm is the same for normal spleen, with or without 0.0018 M calcium chloride, and 14 per cent lower for leukemic spleen when 0.0023 M calcium chloride is used.

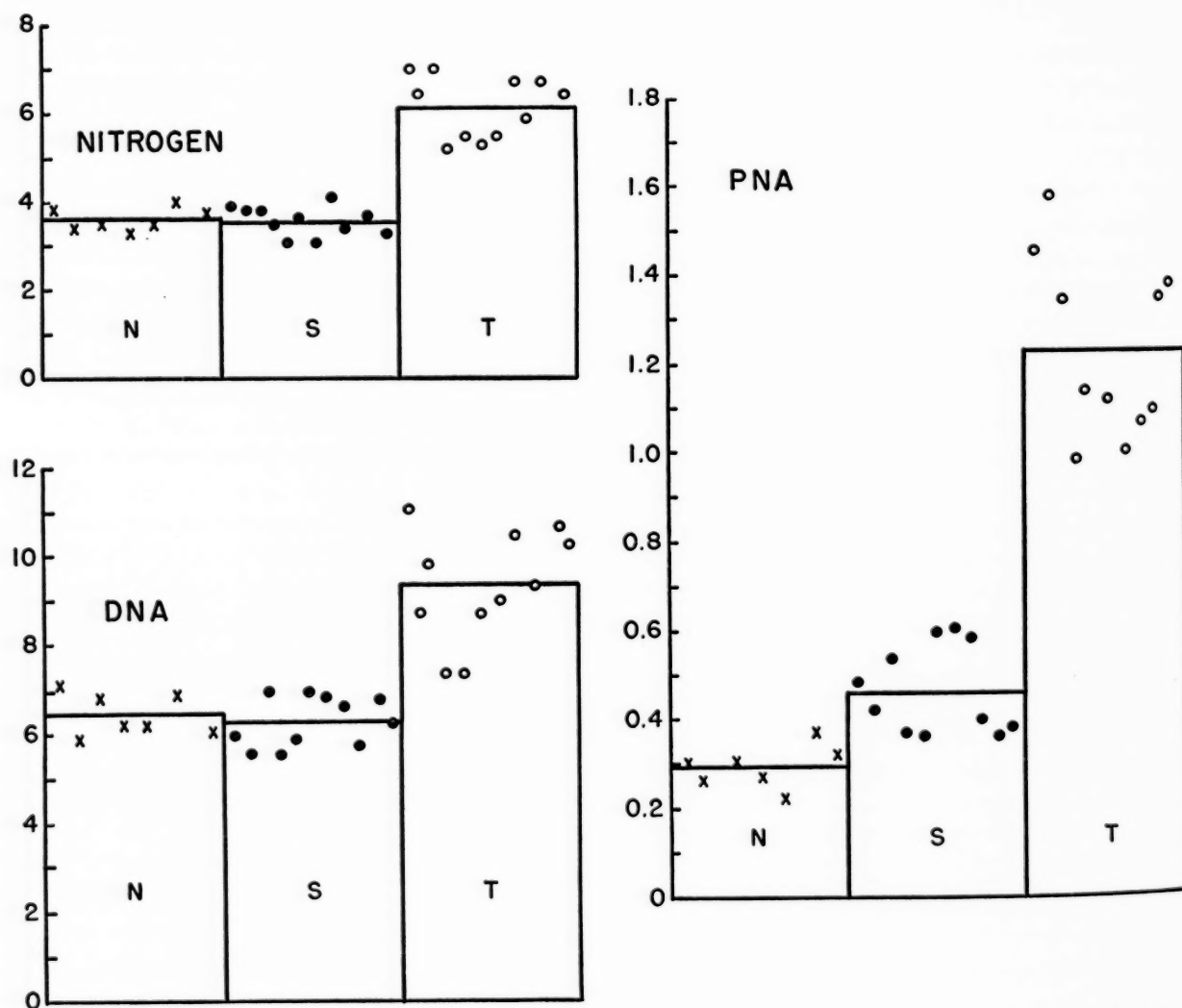


CHART 1.—The nitrogen, DNA, and PNA contents of nuclei isolated from mouse spleen, in mg.  $\times 10^{-9}$  per nucleus. N = normal spleen; S = spontaneous leukemia spleen; and T = transplanted leukemia spleen.

A second suggestion as to whether the nuclei are contaminated with cytoplasmic PNA may be gleaned from a comparison of the isolated nuclei with the whole "nuclear fraction" obtained by one centrifugation in sucrose alone (11). The PNA: nitrogen ratio (0.08) for the isolated normal nuclei is similar to the ratio found on the whole "nuclear fraction," 0.07; while for leukemic spleen the ratio for the isolated nuclei is 0.20, definitely lower than the ratio of 0.26 found for the whole "nuclear fraction." These ratios are also somewhat lower than those found for normal and leukemic spleen nuclei isolated with the aid of citric acid (1).

The reasons for the effect of calcium in preventing stickiness of nuclei have not been investigated in detail. In the experiments on whole spleen reported above, the nuclear fraction from 0.4 gm. of spleen occupied a volume of 0.3 cc. in sucrose with calcium, and of 1.0–1.2 cc. in sucrose alone, after

TABLE 1

THE EFFECT OF CALCIUM CHLORIDE ON THE PNA CONTENT OF MOUSE SPLEEN CYTOPLASM

	NORMAL		LEUKEMIC			
	No Ca	Ca	Exp. 1		Exp. 2	
	No Ca	Ca	No Ca	Ca	No Ca	Ca
Volume of nuclear fraction (cc.)	1.15	0.35	1.00	0.26	1.00	0.30
Volume of cytoplasmic fraction	4.65	5.45	4.60	5.54	4.60	5.00
PNA of cytoplasm (mg. per cc.)	0.22	0.19	0.38	0.27	0.39	0.31
PNA of cytoplasm (total, mg.)	1.02	1.03	1.75	1.50	1.79	1.55

centrifuging 15 minutes at 1,000 g. Only a little more packing is obtained after 30 minutes at 20,000 g. Once they have been packed by centrifugation, even at low speed, the nuclei clump badly and cannot be resuspended. Similar observations have been made by others (1). The calcium prevents this, perhaps by decreasing the solubility of the nucleohistone. Phase contrast photomicrographs of nuclei isolated with the use of calcium show condensed chromatin (15), while in nuclei suspended in 0.88 M sucrose alone the chromatin appears to be evenly dispersed (14). Why a higher concentration of calcium is required for leukemic spleen has not been determined; but it should be noted that when citric acid is used to prevent stickiness of spleen nuclei the leukemic nuclei require more acid than the normal ones (1).

It may be seen (Chart 1) that in transplanted leukemia the DNA per nucleus was markedly increased (to 1.45 times the normal value), while in spontaneous leukemia no such change occurred. It would be of great interest to check these findings

on isolated nuclei by determining the number of nuclei per gram of whole spleen and calculating the DNA per nucleus from the DNA content of the whole tissue, as was done by Price and Laird (13) for liver. So far, however, it has not been found possible to obtain reliable nuclear counts on whole spleen. When the organ is homogenized before the removal of the connective tissue fibers, microscopic examination of the homogenate shows clusters of nuclei trapped in networks of fibers, and reliable nuclear counts have not yet been obtained (15). Without values for DNA per nucleus on whole spleen, there is no check on the loss of DNA during the isolation procedure. That any loss of DNA has taken place during the isolation seems unlikely, however, since the value obtained for normal nuclei,  $6.5 \times 10^{-9}$  mg. per nucleus, is the same as the values found for mouse and rat spleen nuclei isolated in citric acid (15). Nuclear counts on whole spleen would also serve as a measure of the number of cells per unit weight of tissue (13). Since the total DNA is slightly lower in transplanted leukemia (14 mg. per gram) than in normal spleen (15 mg. per gram) (11), the elevated DNA per nucleus found in transplanted leukemia would indicate that the number of cells per gram of spleen may be considerably reduced.

Observations by other workers on the DNA content of nuclei of neoplastic cells are quite variable. In a single experiment on leukemic mouse spleen, normal values were found (1). Increased amounts of DNA were observed by Stowell in leukemic nuclei (17), although Davidson, Leslie, and White (5), studying unspecified types of human leukemia, failed to observe any change in DNA per nucleus. In tumors of the liver the DNA per nucleus is not increased (3, 9, 13); but elevations to 2 and 3 times the normal values have been observed in mouse ascites tumors (7, 8), and a marked elevation is reported for GRCH 15 tumor in the fowl (4).

It is doubtful whether the increase in DNA in transplanted leukemia can be ascribed to a primary neoplastic change; its most probable cause is an increased mitotic rate. An even more striking elevation has been observed in regenerating rat liver by Price and Laird (13), who found that 24 hours after partial hepatectomy the DNA per nucleus was increased to 1.8 times the normal value; up to this time mitoses were not observed. During the second and third days mitoses reached a maximum frequency, and the average DNA per nucleus fell sharply.

An explanation of these findings on a cellular basis is not easily obtained by chemical analysis of mixed populations of nuclei. Cytochemical methods, in which individual nuclei are studied, give

more detailed information. In the pronephros, a tissue of high mitotic rate, of a newly hatched larva of *Ambystoma opacum*, Swift (18) found the normal amount of DNA (Class I) in telophase nuclei; twice this amount (Class II) in prophase; while in interphase the values were distributed over a broad curve between Class I and Class II, with most measurements falling at the lower end. He concludes that "through much of the interphase the nuclei must keep the Class I amount of DNA, since the majority of the measured resting cells have this value. Then, while still appearing microscopically as a resting nucleus, DNA is built up, and, when the doubled amount is reached, prophase is initiated." Similar results were obtained on embryonic mouse liver.

It therefore seems that, in the absence of extensive polyploidy, the average DNA per nucleus is correlated with the mitotic rate of the tissue. Regenerating liver (DNA  $1.8 \times$  normal) triples in weight in about 3 days; in transplanted leukemia (DNA  $1.45 \times$  normal) the spleen triples in weight in about 10 days; while in spontaneous leukemia and in liver tumors, where growth is slow, no increase in DNA is seen.

The increase in the PNA content of the nuclei in the transplanted leukemia to over 4 times the normal value is even more striking. PNA has been found in chromosomes by Mirsky and Ris (10) and by Kaufmann, McDonald, and Gay (6). Isolated residual chromosomes prepared from leukemic mouse spleen by the procedure of Mirsky and Ris contained about twice as much PNA as those from normal spleen (12).

A simple calculation, however, shows that the PNA isolated with the chromosomes can account for only a fraction of that found in the whole nucleus. In the whole chromosomes from normal spleen, the ratio of PNA to DNA was about 0.015.<sup>1</sup> The chromosomes isolated from one nucleus (presumably containing  $6.5 \times 10^{-9}$  mg. of DNA) would therefore have contained  $6.5 \times 0.015 = 0.10 \times 10^{-9}$  mg. of PNA. Since  $0.29 \times 10^{-9}$  mg. per nucleus was found, the excess nuclear PNA not in the chromosomes was  $0.19 \times 10^{-9}$  mg. per nucleus. By the same reasoning, in transplanted leukemia  $9.4 \times 0.03 = 0.28 \times 10^{-9}$  mg. of the nuclear PNA would have been found in the isolated chromosomes; the nuclear PNA not in the chromosomes would be  $1.23 - 0.28 = 0.95 \times 10^{-9}$  mg. per nucleus, a fivefold increase over the normal value.

Since isolated chromosomes contain nucleoli (10), the true PNA content of the chromosomes themselves is probably less than the values found

<sup>1</sup> M. L. Petermann and E. J. Mason, unpublished results.

on chemical analysis; the extra-chromosomal PNA associated with the isolated nuclei may therefore be even greater than these calculations indicate. Also, how much PNA may have been lost during the isolation of the chromosomes cannot be determined. It does appear, however, that the leukemic nuclei contain large amounts of PNA not associated with the chromosomes. This finding is in agreement with results obtained by cytochemical methods; Thorell (19) has shown by ultraviolet absorption that the large and numerous nucleoli characteristic of malignant lymphocytes are rich in PNA. Whether any of this excess PNA, like the DNA, is ascribable to the malignant state, and not just to the rapid growth of the tissue, cannot be

TABLE 2  
THE NITROGEN DISTRIBUTION IN MOUSE SPLEEN NUCLEI  
(Mg. per nucleus  $\times 10^9$ )

	NORMAL		TRANSPLANTED LEUKEMIA	
	Total	N cont.	Total	N cont.
DNA	6.5	0.91*	9.4	1.32*
Histone	6.5	1.17†	9.4	1.69†
PNA	0.29	0.04*	1.23	0.17*
Residual chromosome protein		0.47		0.58
Sum of nitrogen fractions		2.59		3.76
Total nitrogen:		3.60		6.10
Nitrogen not accounted for:		1.01		2.34
Isolated chromosomes:				
DNA:N ratio	2.4		2.6	
Total nitrogen in chromosomes		2.71		3.62
Residual chromosome nitrogen				
by difference		0.53		0.43
16 per cent of total		0.47		0.58

\* 14 per cent of NA.

† 18 per cent of histone.

determined at the present time. Thorell (19), in a careful study of the PNA content of the developing myelocyte, found that in the primitive myeloblast the PNA content of both nucleoli and cytoplasm was elevated. This is strikingly parallel to the present findings in transplanted leukemia. In spontaneous leukemia, where growth and cell division are relatively slow, the PNA is only slightly increased either in the nuclei or in the cytoplasm.<sup>2</sup>

A rough calculation of the nitrogen distribution in normal and transplanted leukemic nuclei is shown in Table 2. The amount of histone has been assumed to equal the amount of DNA. Although this is considerably less histone than the amount found in the chromosomes from calf thymus (10), it is in better accord with the DNA:N ratios found for mouse spleen chromosomes. The residual chro-

<sup>2</sup> M. L. Petermann and A. M. Larack, unpublished results.

mosome nitrogen has been calculated from the DNA:N ratios for whole chromosomes and the percentage of nitrogen recovered in residual chromosomes (12).<sup>3</sup> Approximate as these values are, they indicate the presence of more nonchromosome nitrogen in the leukemic nucleus ( $2.41 \times 10^{-9}$  mg.) than in the normal nucleus ( $1.00 \times 10^{-9}$  mg.). This, also, is in agreement with morphological observation of the frequent large nucleoli present in leukemic lymphocytes.

### SUMMARY

Nuclei have been isolated from the spleens of mice bearing spontaneous or transplanted leukemia.

In spontaneous leukemia, which develops slowly, the PNA per nucleus is increased to 1.6 times the normal amount, and there is no change in DNA or nitrogen.

In transplanted leukemia, which develops rapidly, the PNA, DNA, and nitrogen per nucleus are increased to 4.2, 1.45, and 1.69 times their respective amounts in normal spleen.

The differences in nucleic acid content found in the nuclei from the transplanted leukemia are similar to those reported for rapidly growing tissues, while the nuclei from the spontaneous leukemia more closely resemble the normal nuclei. Thus, the changes seem to be characteristic of rapid growth rather than of any primary neoplastic process.

### ACKNOWLEDGMENTS

The assistance of Roscoe C. Funk, Jr., and of Nancy A. Mizen is gratefully acknowledged.

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# Tracer Studies on the Metabolism of the Gardner Lymphosarcoma

## I. The Uptake of Radioactive Glycine into Tumor Protein\*

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### INTRODUCTION

Since the classic experiments of Schoenheimer (11), the incorporation of isotopically labeled amino acids into body proteins has been used as an indicator of the biological synthesis of protein. The availability of  $C^{14}$  has led to extensive studies on tissue slices and homogenate systems (1, 2), as well as to further experiments employing intact animals. As a result, the nature and mechanism of the incorporation process have been partially clarified. Nevertheless, no conclusions can as yet be drawn as to whether one is measuring *de novo* synthesis of protein or an exchange of amino acids or peptide fragments with those in existing proteins.

In spite of this limitation, the importance of protein synthesis in the field of tumor metabolism provides a compelling reason for undertaking tracer studies on amino acid uptake with tumor tissues. To date, some studies have been conducted on intact tumor-bearing animals (6, 8), but only the papers by Zamecnik and associates (17) and Winnick (15) report that isolated tumor tissues were used as the experimental material.

In the present study, the Gardner lymphosarcoma, 6C3H-ED, of the mouse was employed. That this tissue is particularly advantageous for metabolic studies was pointed out by Summerson, Gilder, and Lee (14). Unlike most tumors, this tissue is free from central necrosis. Moreover, intact tumor cell suspensions can be prepared easily. The characteristics of the process by which glycine-2-

$C^{14}$  is incorporated into the protein of such cell suspensions are reported in the first paper of this series, as are certain control experiments which are intended to minimize the probability that the radioactivity in the protein is the consequence of an artifact process such as adsorption.

### METHODS

*The cell suspension.*—Small pieces of tumor tissue were transplanted subcutaneously into both flanks of C3H mice by the trocar technic. The animals were killed after the tumors had grown for 2 weeks. Tumors from 10 to 15 animals were pooled for each experiment. Suspensions of intact tumor cells were made by gently grinding the tissue in an aluminum cylinder, one end of which contained a Monel metal screen (60 mesh). The cells were washed through the screen into small beakers with the aid of the Krebs-Ringer-Phosphate (KRP) solution described below. The cells were then centrifuged for 5 minutes in a clinical centrifuge, after which procedure the supernatant fluid was discarded. The packed cells were diluted to an appropriate volume with the KRP solution (usually 1 volume of cells to 2 volumes of medium, giving about  $300 \times 10^6$  cells per milliliter). Aliquots of this suspension, usually 0.5 ml., were added to each incubation flask, along with the radioactive amino acid. KRP solution was then pipetted into the flask so as to make a final volume of 1 ml. When other metabolites, such as glucose, were added, the KRP solution was reduced so that the final volume remained at 1 ml. Smears of the cells were routinely made with Wright's stain to determine whether the cells were intact. Cell counts were made by counting diluted suspensions of the tumor cells in a hemocytometer. For this purpose, the cells were stained with methylene blue in 1 per cent acetic acid solution.

*Medium.*—The medium in which the cells were suspended consisted of a modified Krebs-Ringer-Phosphate solution (KRP). It was prepared by mixing stock solutions of 19.1 ml. of 4.5 per cent NaCl, 2 ml. of 5.75 per cent KCl, 0.4 ml. of 6.1 per

\* Aided by research grants from the American Cancer Society, recommended by the Committee on Growth of the National Research Council, and the National Cancer Institute, Public Health Service.

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Received for publication February 6, 1951.

cent  $\text{CaCl}_2$ , and 0.1 ml. of 19.1 per cent  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . The mixture was diluted to a volume of 108 ml., and 90 ml. of this was added to 10 ml. of 0.1 M sodium phosphate buffer, pH 7.4. In later experiments, a solution designated as KRPB was used and was prepared from the KRP solution by reducing the NaCl to 16 ml., and using only 5 ml. of the phosphate buffer plus 5 ml. of water so that the final phosphate buffer concentration was 0.005 M. Solid  $\text{NaHCO}_3$  (210 mg.) was added, and the solution was gassed with 95 per cent  $\text{O}_2$ -5 per cent  $\text{CO}_2$ . The KRP solutions were equilibrated with 100 per cent  $\text{O}_2$ .

**Incubation procedure.**—The tumor cells were incubated with appropriate metabolites in 25-ml. glass-stoppered flasks in a constant temperature bath at 38° C. A shaking rate of 100 cycles per minute was maintained. In later experiments, the Dubnoff metabolator (5) was used so that it was possible to maintain a constant gaseous atmosphere of 95 per cent  $\text{O}_2$ -5 per cent  $\text{CO}_2$ . At the conclusion of the incubation, the flask contents were inactivated with an equal volume of 10 per cent trichloroacetic acid (TCA).

**Washing, plating, and counting the protein.**—The TCA insoluble material was washed and the lipid extracted as described by Winnick *et al.* (16). Nucleic acid was removed according to the procedure of Schneider (10). The resulting pellets of protein were homogenized in a petroleum ether-ether-acetone solution (6:3:1/2). This suspension was then plated onto tared aluminum discs and evaporated to dryness in air. The protein was then heated in an oven at 95° C. for 1 hour and equilibrated in air for a second hour, after which the weight of the protein on the plates was determined. The plates were counted with a thin mica end-window Geiger-Müller tube attached to a Tracer-lab autoscaler. The efficiency of the counter was 8 per cent, and the counting was continued for a long enough time to obtain an accuracy of  $\pm 3$  per cent. The specific activity of the protein was corrected for self absorption from an empirically determined curve. The values recorded in the tables are the means of triplicate flasks except where simultaneous measurements of oxygen consumption were made. In the latter case, the experiments were performed in duplicate. The coefficient of variation was generally about 5 per cent.

The radioactive data are expressed either as specific activity in counts per minute per milligram of protein (cpm)<sup>1</sup> or as incorporation in  $\mu\text{g C}^{14}/\text{gm}$

<sup>1</sup> The data required to calculate incorporation in  $\mu\text{g C}^{14}$  per gram protein were not determined until this investigation was well advanced. Therefore, certain of the results can only be given accurately in counts/min/mg of protein.

protein.<sup>2</sup> The latter is calculated from the formula:

$$\text{Incorporation} = \frac{\text{counts/min/gm protein}}{\frac{\text{MW of A}^*}{12} \cdot \text{counts/min/}\mu\text{g of A}^*},$$

where A\* represents the original  $\text{C}^{14}$ -labeled amino acid.

## RESULTS

**Characteristics of the plated protein.**—The plated protein contained 12.35 per cent N but no P, as determined by elementary analysis. It was ribose-negative, as evidenced by the qualitative Bial test (7), but after 24 hours in 5 per cent thymol solution showed a faint pink (7), indicating the presence of some carbohydrate. This carbohydrate material and moisture probably account for the low N value which was found. The specific activity of the protein was not changed by incubating it for 3 hours in water at 90° or in 0.1 M NaOH for 2.5 hours at 23°. The specific activity of the protein was also unchanged after treatment with mercaptoethanol or performic acid (9), indicating that the counts were not concentrated in small peptide fragments bound to the protein by disulfide linkages.

**Effect of various treatments on glycine uptake into protein.**—When the tumor cells were subjected to various treatments which tended to destroy them, the uptake of  $\text{C}^{14}$ -labeled glycine into the tumor protein was grossly inhibited. Data illustrating this point are shown in Tables 1, 2, and 3.

It may be seen (Table 1) that the addition of TCA to the incubation flasks at the start of an experiment reduced the counts in the protein to a negligible quantity. This also occurred when the cells were quickly frozen at a temperature of dry ice plus acetone. Cells homogenized in the Potter-Elvehjem apparatus or subjected to the shearing forces of the Waring Blendor also exhibited markedly inhibited uptake. From Table 1 it may also be seen that anaerobiosis inhibited the uptake process 80 per cent when glucose was present and over 90 per cent when glucose was absent. It was found, however, that the incorporation of glycine into cell protein proceeded as well in an atmosphere of 20 per cent  $\text{O}_2$  (air) as in 100 per cent  $\text{O}_2$ .

Table 2 illustrates the effect of increasing hypotonicity on the uptake process. Part of the NaCl of the KRPB medium was replaced in this experiment with water. It may be observed that as hypotonicity increased the specific activity of the protein decreased, and that when the medium in which the cells were suspended was replaced en-

<sup>2</sup> In this expression  $\text{C}^{14}$  merely designates radioactive carbon and is not intended to mean that the incorporation represents  $\text{C}^{14}$  solely.

tirely by distilled water the uptake was almost completely abolished.

Shaking tumor cells for several hours at 38° is a process which may be expected to speed up the destruction of essential metabolic systems within the cells. Lymphosarcoma cells or cell suspensions from the spleens of normal animals aged in this way showed markedly reduced uptake of either

glycine or alanine into cell proteins (Table 3). When the tumor cells were kept at 4° for 2 hours prior to the start of an incubation, however, the rate of uptake of glycine or alanine into the protein was not changed.

*Effect of changes in the ionic composition of the medium on uptake.*—To arrive at a medium optimal for glycine uptake, the  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ , and  $\text{K}^+$  ion concentrations of the medium were varied, and the effect of these changes on the glycine uptake into the protein was determined. When the concentration of one of these ions was decreased, the concentration of  $\text{Na}^+$  ion was correspondingly increased and vice versa.

It was found that concentrations of  $\text{Ca}^{++}$  from 0 to  $2.7 \times 10^{-3}$  M added to the medium did not affect the specific activity of the protein incubated with glycine-2- $\text{C}^{14}$ . Concentrations of  $\text{Mg}^{++}$  of 0 to  $9 \times 10^{-4}$  M also had no marked effect on either glycine uptake or the respiration of the tumor cells. When the KCl was completely replaced by NaCl, the uptake of glycine was inhibited 45 per cent, but when KCl was present in the medium in concentrations ranging from  $6.4 \times 10^{-3}$  M to  $19.2 \times 10^{-3}$  M, there was no difference in the glycine uptake from that of the control flasks in which the  $\text{K}^+$  concentration used was 0.0128 M. When all of the  $\text{Na}^+$  of the medium were replaced by  $\text{K}^+$ , there was a 54 per cent inhibition of glycine uptake.

*Effect of variation of pH.*—The effect of variations in the initial pH of the medium was also studied. Variations in the initial pH of between 7 and 8 had no marked effect on glycine uptake (Table 4), and pH 7.4 was the apparent optimum pH for the medium; there was a 45 per cent inhibition at pH 6.5. During the course of an experiment in which glucose was present, the pH of an experimental flask usually dropped to 6.4. Uptake in the absence of glucose was much less affected by changes in this pH region.

The rate of glycine uptake over a long incubation period was not always maintained at a linear rate with time. This is partly attributable to the inhibitory effect of the low pH which resulted. In experiments in which the phosphate buffer concentration was increased while the initial pH was maintained at 7.4 (Table 5), it was observed that, although the pH of the flasks was stabilized, the higher phosphate concentrations progressively inhibited glycine uptake. In contrast to this, when bicarbonate buffer replaced phosphate buffer, glycine uptake into the protein was stimulated. Consequently, concentrations of 0.005 M phosphate and 0.025 M bicarbonate were chosen to buffer the medium in subsequent experiments.

TABLE 1  
EFFECT OF VARIOUS TREATMENTS ON  
GLYCINE UPTAKE OF TUMOR\*

Treatment	cpm†
Control‡	205
TCA added at start of incubation	0.5
Control	69
Homogenized 3 min.	19
Control	91
Frozen at acetone-dry ice temp., thawed after 5 min.	1
95 per cent $\text{O}_2$ -5 per cent $\text{CO}_2$ , 0.025 M glucose (control)	35§
95 per cent $\text{N}_2$ -5 per cent $\text{CO}_2$ , 0.025 M glucose	7
95 per cent $\text{O}_2$ -5 per cent $\text{CO}_2$ , 0.025 M pyruvate	15
95 per cent $\text{N}_2$ -5 per cent $\text{CO}_2$ , 0.025 M pyruvate	1
95 per cent $\text{N}_2$ -5 per cent $\text{CO}_2$ , 0.025 M lactate	1

\* 0.002 M Glycine-2- $\text{C}^{14}$  (3.45  $\mu\text{g}/\text{mg}$ ) were in each flask; temp. 38° incubated 100 min.

† Counts/min/mg.

‡ 0.6 mM glycine-2- $\text{C}^{14}$  used, incubated 60 min.

§ Radioactivity in  $\mu\text{g}$   $\text{C}^{14}/\text{gm}$  protein in this series of experiments.

TABLE 2  
EFFECT OF HYPOTONICITY OF THE  
MEDIUM ON THE GLY-  
CINE UPTAKE\*

Exper.	$\text{Na}^+$ conc. mm/l	cpm
1	131 (control)	91.2
	99	79.9
	73	58.4
2	131 (control)	169
	0†	12.3

\* KRPB medium used. Where  $\text{Na}^+$  ion conc. was lowered from the control level, water replaced part of the NaCl. Incubation time, 100 min.; temp., 38°. Gas phase, 95 per cent  $\text{O}_2$ -5 per cent  $\text{CO}_2$ . Flask contents: 0.1 ml. of 0.025 M glycine-2- $\text{C}^{14}$  (3.45  $\mu\text{g}/\text{mg}$ ), 0.2 ml. of 2 per cent glucose solution, 0.5 ml. tumor suspension, 0.2 ml. KRPB, to give final volume of 1 ml. Each ml. packed cells diluted to 3 ml. to make cell suspension.

† Cells suspended in 2 volumes  $\text{H}_2\text{O}$  for 15 minutes.

TABLE 3  
EFFECT OF AGING CELLS UPON AMINO ACID UPTAKE\*

Amino acid	Tissue	Treatment	$\mu\text{g. C}^{14}$ gm. protein
Glycine-1- $\text{C}^{14}$ 3.7 $\mu\text{g}/\text{mg}$	Spleen†	Control	43.2
		Aged 1 hr. at 38°	24.1
		Aged 2 hr. at 38°	17.1
DL-alanine-1- $\text{C}^{14}$ 3.6 $\mu\text{g}/\text{mg}$	Tumor	Control	7.9
		Aged 2 hr. at 38°	0.63

\* KRPB solutions 95 per cent  $\text{O}_2$ -5 per cent  $\text{CO}_2$ . Flask contents: 0.1 ml. amino acid (0.001 M final conc.), 0.5 ml. cell suspension, 0.4 ml. KRPB solution. Incubated 100 minutes.

† The suspension of spleen cells was prepared essentially in the same manner as the lymphosarcoma cells.

*Effect of glycine concentration.*—Table 6 indicates that, as the glycine concentration was increased, the rate of uptake increased in a linear fashion until a glycine concentration of about 0.008 M was achieved. Above this level, increasing

high cell concentrations the available glycine was a limiting factor for uptake. Therefore, an experiment was performed in which the glycine concentration was raised to 0.002 M. Under these conditions the rate of uptake of glycine was linear for 100 minutes, and the specific activity of the dilute was the same at each time point as that of the concentrated cell suspension (Table 7).

*Glycine uptake with and without glucose as a function of time.*—Table 6 shows that the rate of uptake of glycine into the protein is constant for 100 minutes. In this experiment, glucose was present in the incubation medium. When glucose was not added to the incubation medium, the rate of glycine uptake was not so rapid nor was the rate linear after the first 40 minutes. The capacity of glucose to stimulate amino acid uptake is discussed in more detail in paper II.

The stimulation of amino acid uptake owing to the presence of glucose has been attributed to the increased energy made available through glycolysis. This energy could be directed towards an increased rate of peptide bond formation. However,

TABLE 4  
THE EFFECT OF VARYING THE INITIAL  
pH ON THE UPTAKE OF GLYCINE  
INTO THE PROTEIN OF TUMOR SUS-  
PENSIONS\*

Initial pH of KRP	Final pH†	cpm
6.5	6.25	29.6
7.0	6.40	50.7
7.4 (control)	6.35	54.1
8.0	6.42	47.0

\* Each flask contained 0.50 ml. tumor suspension ( $170 \times 10^4$  cells), 0.10 ml. glycine-2- $C^{14}$  (0.67 mm/1, 3.45  $\mu$ c/mg), 0.10 ml. 2 per cent glucose, and KRP of the indicated pH to make a final volume of 1 ml. Incubation time, 1 hour; temperature, 38°.

† Measured with the glass electrode after adding 1 ml. distilled water.

TABLE 5  
EFFECT OF BUFFER AND BUFFER CAPACITY  
ON GLYCINE UPTAKE

Buffer Conc. M	Final pH*	cpm
0.01 Phosphate (control)	6.30	163
0.05 Phosphate	7.05	113
0.10 Phosphate	7.15	77
0.01 $HCO_3$	6.40	250
0.01 Phosphate (control)	6.35	54.1
0.01 Phosphate plus	6.90	73.0
0.025 $HCO_3$		

\* Measured with the glass electrode after adding 1 ml.  $H_2O$ ; 0.670 mm/1 glycine-2- $C^{14}$  (3.45  $\mu$ c/mg) used; final flask volume, 1 ml.; glucose conc., 0.2 per cent; incubation time, 1 hour; temp. 38°.

the glycine concentration produced no further increase in the specific activity of the protein. Glycine concentrations as high as 0.0033 M have been studied. It may also be observed (Table 6) that the presence of glycine did not increase the net oxygen uptake of the tumor cells. This will be discussed further in the following paper.

*Effect of cell concentration on uptake.*—Experiments were performed in which all components of the flasks were held constant except the number of cells in each flask. When the incubation proceeded for 2 hours, the specific activity of the protein from flasks in which the concentration of cells was dilute was markedly higher than from the flasks in which the cell concentration was greater. A similar effect has been reported by Borsook (3) on the lysine uptake into guinea pig liver homogenates. This tendency for the more dilute cell suspensions to show a higher specific activity seemed to increase as the incubation time progressed, being least at the shorter incubation periods. This suggested that at

TABLE 6  
EFFECT OF GLYCINE-2- $C^{14}$  CON-  
CENTRATION ON UPTAKE OF GLY-  
CINE AND OXYGEN

Glycine conc. M	$\mu$ g. $C^{14}$ gm. protein	$\mu$ l. $O_2$
0.0016	37.3	79
0.0008 (control)	41.7	79
0.0003	19.5	72
0.00016	11.5	74
0		75

\* Final volume, 1.3 ml; incubated 70 minutes at 37°, flasks flushed with 100 per cent  $O_2$ . KRP solution was used; 0.015 M glucose in all flasks. Activity of glycine used was 3.45  $\mu$ c/mg.

TABLE 7  
EFFECT OF CELL CONCENTRATION AND  
TIME ON GLYCINE UPTAKE  
INTO TUMOR\*

Incubation time (min.)	ml. cells in final vol. of 0.9 ml. (ml.)	cpm
30	0.3	49.5
"	0.6 (control)	49.4
60	0.3	118
"	0.6 (control)	108
100	0.3	185
"	0.6 (control)	169

\* 8.4 ml. packed cells diluted to 16.8 ml. with KRPB to make cell suspension. Eighteen C3H females were used, 14 days after transplanting; gassed with 95 per cent  $O_2$ -5 per cent  $CO_2$ , shaken at 90 cycles per minute; temp., 38°; 0.025 M glucose, 0.002 M glycine-2- $C^{14}$  (3.45  $\mu$ c/mg) in each flask.

since glycine- $C^{14}$  may be converted into serine- $C^{14}$  (12), the presence of glucose could conceivably stimulate this amino acid interconversion process rather than the amino acid uptake process.

To test this point, radioactive protein was hy-

drolyzed and radioactive serine isolated by the carrier technic (12). The tosyl derivative was made, recrystallized, and counted. This was done for tumor protein and also for protein from the cells of normal spleen tissue which had been incubated with glycine both in the presence and absence of glucose. For both tissues, the percentage of all the counts in the protein which could be attributed to serine- $C^{14}$  was about 20 per cent, and this was not changed in the protein from cells in which the synthesis of protein was stimulated by glucose. The results therefore suggested that the presence of glucose may indeed have stimulated the conversion of glycine to serine, but that if it did so it also stimulated the incorporation of both glycine and serine into cell protein to an equivalent extent.

*Effect of metabolic inhibitors.*—A cyanide and azide concentration of 0.005 M and a dinitrophenol concentration of 0.0005 M inhibited glycine- $C^{14}$  uptake 94, 98, and 99 per cent, respectively. The effect of other metabolic inhibitors and of other metabolites are reported in paper II. The results emphasize the importance of oxidation reactions for protein synthesis by this tumor. As evidenced by the data of Table 1, some incorporation can occur in these cells under  $N_2$  when glucose is added to the medium, but whether  $O_2$  has been completely excluded is always a moot question in an experiment of this kind.

#### DISCUSSION

In all experiments with radioactive isotopes, the investigator is concerned with whether or not he has removed all the radioactive impurities. This is of particular importance in an experiment in which a small molecule such as an amino acid is incubated with a bulky, heterogeneous substance such as protein. The data reported in this paper make it abundantly clear that adsorption of radioactive impurities due to incomplete washing cannot account for the experimental results. As is evident from the data already reported and from the data of paper II, the uptake process depends upon the integrity of metabolic processes within the cell. Similar observations have also been reported by workers who have investigated other tissues, using glycine, alanine, and other amino acids (1, 13).

It may also be pointed out that the recent studies by Borsook (4) on rabbit bone marrow cells are in agreement with the data reported in this paper on mouse lymphosarcoma cells with respect to the characterization of the uptake process.

#### SUMMARY

The incorporation of glycine-2- $C^{14}$  into the protein of the Gardner lymphosarcoma cells has been

studied. The process is not markedly affected by changes in the  $Ca^{++}$ ,  $Mg^{++}$ , or  $K^{+}$  ion concentrations of the medium or by changes in the hydrogen ion concentration between pH 7 and 8. Below pH 6.5, the uptake is inhibited. Glycine uptake is stimulated by glucose and by bicarbonate and, in the presence of these, is linear for 2 hours. Metabolic inhibitors or conditions whereby the cells are disrupted almost completely abolished uptake.

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# Tracer Studies on the Metabolism of the Gardner Lymphosarcoma

## II. Energy-yielding Reactions and Amino Acid Uptake into Protein of the Tumor Cell\*

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### INTRODUCTION

Formation of peptide bonds in protein synthesis is an energy-requiring reaction. The necessary energy, presumably, can be derived from either the reactions of glycolysis or aerobic oxidation. It is widely accepted that the energy derived from metabolic processes is stored and transferred through the synthesis of compounds with high energy phosphate bonds, particularly adenosine triphosphate (ATP). It is to be noted that only *two* such bonds are formed from one molecule of glucose by glycolysis, whereas at least 30 (7) are formed per molecule of glucose by oxidation via the Krebs' tricarboxylic acid cycle. In view of these facts, it was particularly pertinent to investigate the relation of these metabolic processes to the activity of amino acid incorporation into the tumor protein.

### METHODS

The procedures employed in this investigation and the methods for measuring amino acid uptake are described in paper I (6). Oxygen utilization was measured according to conventional methods (11).

*Demonstration of  $C^{14}$  in respiratory  $CO_2$ .*—To demonstrate the oxidation of radioactive amino acids or acetate to  $CO_2$ , the respiratory  $CO_2$  was trapped in 20 per cent KOH in the center well of the Warburg flask. At the conclusion of the incubation, 1 ml. of 10 per cent trichloroacetic acid was added through a side arm, and the flasks were al-

lowed to stand for an additional 30 minutes. The alkaline solution from the center well was then washed into a glass-stoppered flask which contained 5 ml. of 0.1 M  $Ba(OH)_2$ . After standing overnight, the precipitate (about 1 mg.) of  $BaCO_3$  was filtered onto No. 50 Whatman filter paper and counted with a thin mica end-window Geiger-Müller tube.

*Citrate determination.*—Citric acid was determined by the method of Natelson *et al.* (8). The color was read with a Beckman Model B spectrophotometer at 450 m $\mu$ . A standard curve consisting of three points was determined with every group of unknowns. Normal heptane was used to extract the pentabromacetone.

### RESULTS

*Glycolysis and amino acid uptake.*—When radioactive amino acids were incubated in the presence of glucose, the tumor cells consistently incorporated more radioactivity into their protein than in the absence of glucose. Table 1 illustrates this fact when alanine, glycine, leucine, or phenylalanine is the labeled amino acid used. The presence of the glucose, however, did not increase the net oxygen uptake of the tumor cells. The apparent 48 per cent stimulation of oxidation due to glucose in the case of the leucine experiment occurred because the respiration of the cells continued for a time in the presence of glucose after the control cells had ceased to respire. The rates of respiration were, however, equal.

Summerson *et al.* (10) have also found that the presence of glucose does not increase respiration by the Gardner tumor cells, and chemical determinations by these workers have demonstrated that the glucose consumed can be substantially accounted for as lactic acid.

*Inhibitors of glycolysis.*—The role of arsenate, iodoacetate, and fluoride as glycolytic inhibitors is well known. It may be observed (Table 2) that these substances markedly inhibited glycine up-

\* Aided by research grants from the American Cancer Society, recommended by the Committee on Growth of the National Research Council, and the National Cancer Institute, Public Health Service.

† Abraham Rosenberg Research Fellow, 1949–50; Public Health Service Research Fellow of the National Cancer Institute, 1950–51. The work reported was taken from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry at the University of California.

take by the tumor cells. The inhibition of respiration paralleled the inhibition of glycine uptake due to arsenate, but, while glycine uptake was inhibited 96 per cent by 0.001 M fluoride, oxygen uptake was inhibited only 51 per cent.

**Pyruvate.**—Although the addition of pyruvate to flasks containing the tumor cells but no other substrate stimulated oxidation, the effect on amino

TABLE 1  
EFFECT OF GLUCOSE ON AMINO ACID UPTAKE  
AND RESPIRATION\*

Amino acid	Glucose conc. M	$\mu\text{g. C}^{14}\dagger$ gm protein	O <sub>2</sub> uptake ( $\mu\text{l.}$ )	Incub. time (min.)
None	0		157	60
Glycine (control)	0	17.1	135	"
"	0.02	24.4	102	"
None (control)	0		208	80
None	0.022		212	"
Alanine (control)	0	5.26 $\dagger$		100
"	0.02	23.2 $\dagger$		"
Leucine (control)	0	33.5	117	180
"	0.01	85.1	173	"
None	0		114	"
None	0		137	"
Phenylalanine (control)	0	14.6	122	"
"	0.01	22.1	152	"

\* Glycine-2-C<sup>14</sup> (3.45  $\mu\text{c}/\text{mg}$ ), DL-leucine-2-C<sup>14</sup> (1.55  $\mu\text{c}/\text{mg}$ ), and DL-phenylalanine-3-C<sup>14</sup> (2.48  $\mu\text{c}/\text{mg}$ ) present at 1 mm/l concentration DL-alanine-1-C<sup>14</sup> (3.6  $\mu\text{c}/\text{mg}$ ) at 2mm.

$\dagger$  See paper 1 (6) for explanation of this quantity.

$\ddagger$  Expressed as cpm.

acid uptake was not pronounced. When glycine was the amino acid used, the uptake was only increased by 18 per cent after a 3-hour incubation, while, in the case of alanine, the amino acid uptake was inhibited 45 per cent. The latter may be attributed to the dilution of radioactive alanine by the alanine formed from nonradioactive pyruvate.

**Oxidation of tricarboxylic acid cycle metabolites.**—Of the tricarboxylic acid cycle metabolites, citrate,  $\alpha$ -ketoglutarate, succinate, malate, fumarate, and oxalacetate were tested for their effect on respiration and amino acid uptake by the Gardner cells. Only succinate caused a marked reproducible stimulation of respiration (Table 3). Concomitant with stimulation of respiration there was observed a stimulation of glycine and leucine uptake into the protein. Phenylalanine uptake was stimulated only slightly, and, in the case of alanine, no stimulation by succinate was observed. This last may also be the consequence of increased oxidation and dilution of the labeled alanine. The results with malate were suggestive but not marked.

**Effect of tricarboxylic acid cycle inhibitors.**—From Table 4 it may be seen that fluoroacetate, arsenite, and malonate inhibited the respiration of

the tumor cells and, concurrently with this, a marked inhibition of amino acid uptake was observed. The presence of succinate partially reversed the inhibitory effect of malonate. It is to be noted that powerful inhibitions on glycine uptake were exerted by arsenite and fluoroacetate, although glucose was present in the medium. Glycine uptake is also markedly inhibited by cyanide, azide, dinitrophenol, and anaerobiosis (6).

**The effect of fatty acids.**—The effects of stearate, octanoate, butyrate, propionate, and acetate on glycine uptake were studied. None of these metabolites greatly affected the uptake of glycine, nor did 0.01 M acetate markedly affect alanine uptake. At a concentration of 0.001 M, octanoate had no effect on respiration and proved to be mildly inhibitory for phenylalanine, leucine, and alanine uptake as well.

**Experiments with acetate-1-C<sup>14</sup>.**—The failure to demonstrate increased O<sub>2</sub> uptake with glucose, fatty acids, and most of the Krebs' cycle metabolites by the tumor cell raised the question whether the manometric method provides an adequate measure of oxidation. The presence of the metabolite may not increase oxidation sufficiently for measurement by this technic or there may occur a decrease in the endogenous respiration, so that the net oxygen uptake is unaltered. C<sup>14</sup>-labeled acetate offered a direct method of investigating the me-

TABLE 2  
EFFECT OF INHIBITORS ON RESPIRATION  
AND GLYCINE UPTAKE\*

Exp. conc. mm/l	Addition	$\mu\text{g. C}^{14}$ gm. protein	O <sub>2</sub> uptake ( $\mu\text{l.}$ )
1	None $\dagger$ (control)	17.4	220
10	Arsenate	3.74	62
1	Fluoride	0.77	108
2	None	69.0 $\ddagger$	
1	Iodoacetate	5.1	
0.25	Iodoacetate	12.6	

\* KRP in respiration experiments, otherwise KRPB. Glycine-2-C<sup>14</sup> (3.45  $\mu\text{c}/\text{mg}$ ) used in all experiments. Experiment 1: 1 mm/l glycine, incubated 2 hours at 37°; glucose conc., 0.02 M. Experiment 2: 2.5 mm/l glycine and glucose (0.02 M) incubated 100 minutes at 38°.

$\dagger$  No glucose added.

$\ddagger$  Expressed as cpm in this series.

tabolism of this substance by the tumor cells. An experiment was performed with both suspensions of cells from the spleen<sup>1</sup> of normal C3H mice and tumor cells in the presence and absence of fluoroacetate (Table 5).

It may be observed that both cells oxidized acetate to CO<sub>2</sub> and that fluoroacetate inhibited the respiration of the cells of both tissues. There was

<sup>1</sup> The preparation of the spleen suspensions is described in paper III of this series.

also an inhibition of the production of  $C^{14}O_2$  by the fluoroacetate.

Part of the  $C^{14}$  of the acetate was found in the protein of the tumor cells and in the cells from the spleen. This conversion was also inhibited by

TABLE 3

EFFECT OF TRICARBOXYLIC ACID CYCLE METABOLITES ON RESPIRATION AND AMINO ACID UPTAKE\*

Amino acid	Substrate conc. 0.01 M	$\mu g. C^{14}$ gm. protein	$O_2$ uptake ( $\mu l.$ )
Leucine	None (Control)	33.5	117
"	Citrate	31.0	102
"	Succinate	44.8	226
"	Malate	38.9	136
Phenylalanine	None (control)	14.6	122
"	Citrate	16.8	149
"	Succinate	16.5	253
"	Malate	17.1	121
Glycine†	None (control)	18.3	98
"	Succinate	25.6	242
Glycine‡	None (control)	17.9	57
"	Malate	19.2	69
Glycine§	None (control)	17.1	135
"	$\alpha$ -ketoglutarate	9.4	102
Alanine§	None (control)	27.8	103
"	Succinate	27.3	135

\* DL-Leucine-2- $C^{14}$  (1.55  $\mu c/mg$ ), DL-phenylalanine-3- $C^{14}$  (2.48  $\mu c/mg$ ), and glycine-2- $C^{14}$  (3.45  $\mu c/mg$ ) present at 1 mm/l concentration. DL-Alanine-1- $C^{14}$  (3.6  $\mu c/mg$ ), 3 mm/l. Incubated 3 hours at 37°, KRP solution flushed with 100 per cent  $O_2$ .

† Incubation time, 3.5 hours.

‡ Incubation time, 2 hours.

§ Incubation time, 1 hour.

|| 0.02 M substrate concentration.

TABLE 4

EFFECT OF TRICARBOXYLIC ACID CYCLE INHIBITORS ON AMINO ACID UPTAKE\*

Exp.	Amino acid	Conc. mm/l	Addition	$\mu g. C^{14}$ gm. protein	$O_2$ uptake ( $\mu l.$ )
1	Glycine		None (control)	16.0	110
	"	20	Glucose plus fac†	7.8	42
2	"	10			
	"		None (control)	17.4	220
	"	20	Glucose	30.3	146
	"	20	Glucose plus fac	6.46	75
	"	15			
	"		Glucose plus fac	6.20	62
3	"	20			
	"		Glucose (control)	144†	
	"	25	Glucose plus arsenite	4.8‡	
4	"	5			
	"		$\alpha$ -ketoglutarate (control)	9.4	102
	"	20	$\alpha$ -ketoglutarate plus arsenite	0.58	21
5	"	2.5			
	"		None (control)	18.3	98
	"	10	Malonate plus succinate	11.6	152
	"	10	Malonate	4.92	80
	Alanine		None	8.14	115
	"	10	Malonate	3.54	80

\* Glycine-2- $C^{14}$  (3.45  $\mu c/mg$ ). Exps. 1, 2, 4; (1mm/l); Exp. 3; (2.5 mm/l). DL-Alanine-1- $C^{14}$  (3.6  $\mu c/mg$ ), (1 mm/l). Incubation time: Exps. 1 and 4, 1 hr.; exp. 2, 2 hrs.; exp. 3, 1.66 hr.; exp. 5, 3.5 hr.

† fac is fluoroacetate.

‡ Expressed as cpm.

fluoroacetate. When trichloroacetic acid was added at the start of the incubation, there was no respiration and no  $C^{14}$  in the protein or the respiratory  $CO_2$ .

**Formation of citrate.**—When acetate and oxalacetate were incubated with normal spleen cells or with lymphosarcoma cells, citrate was formed. Results illustrating this fact are shown in Table 6.

TABLE 5

METABOLISM OF ACETATE-1- $C^{14}$  IN TUMOR TISSUE AND NORMAL SPLEEN\*

Fac† conc. mm/l	$\mu g. C^{14}$ gm. protein	$O_2$ uptake ( $\mu l.$ )	Per cent added counts in respiratory $CO_2$
Tumor 6C3HED			
0	0.31‡	3‡	0.02
0 (control)	50.3	211	13
20	12.1	159	5
Normal Spleen			
0 (control)	120	305	30
20	21.1	169	7

\* Flask contents: 0.5 ml. cell suspension ( $155 \times 10^6$  tumor cells or  $200 \times 10^6$  spleen cells), 0.1 ml. of 0.04 M acetate-1- $C^{14}$  (12.3  $\mu c/mg$ ), 0.1 ml. KRP. Incubated 3 hours at 37°. Flasks flushed with 100 per cent  $O_2$ . Six C3H males, weighing about 23 gm., used 13 days after transplanting tumors. Ten normal C3H males weighing about 23 gm. used for normal spleen. Each ml. packed cells diluted with KRP to 3 ml. to make cell suspension.

† Fac is fluoroacetate.

‡ TCA added at zero time.

TABLE 6

CITRATE FORMATION FROM ACETATE AND OXALACETATE BY SUSPENSIONS OF NORMAL AND MALIGNANT CELLS\*

Exp.	Tissue	Incubation time (hr.)	Fac† conc. M	Citrate‡ ( $\mu g.$ )
1	Normal spleen	0	None	18
	"	2	None	18
	"	2	0.02	73
2	"	0	None	12
	"	2	None	19
	"	2	0.02	37
3	Tumor	0	None	20
	"	2	None	72
	"	2	0.02	75
4	"	0	None	24
	"	2	None	88
	"	2	0.02	96

\* Incubated at 22°; about  $240 \times 10^6$  cells suspended in KRPB medium per flask; 5 mm of acetate and 2 mm oxalacetate present.

† Fac is fluoroacetate.

‡ Average of duplicate determinations.

It may be observed that in the case of the normal cells from the spleen, appreciable citrate accumulated only when fluoroacetate was added to the incubation medium. This implies that normally the rates of citrate formation and utilization are in balance. In the tumor, however, citrate accumulated after a 2-hour incubation with its precursors, even in the absence of fluoroacetate. The addition of fluoroacetate induced only a slightly higher accumulation. This suggests that under these conditions citrate synthesis exceeds utilization by this tumor cell.

**Oxidation of amino acids.**—The manometric measurements likewise did not demonstrate the oxidation of amino acids by the tumor cells (Table 7). Nevertheless, when the respiratory  $\text{CO}_2$  was collected and tested for radioactivity, it was clear that the carboxyl groups of glycine and alanine were metabolized to  $\text{CO}_2$ . The oxidation of alanine occurred at a rather rapid rate. The  $\text{C}^{14}$  from  $\beta$ -labeled leucine was also found in the respiratory  $\text{CO}_2$ . This implies a deamination, decarboxylation, and beta oxidation of the resulting 5-carbon fragment (3). The 2-carbon compounds formed from these reactions presumably were oxidized via the Krebs' cycle to  $\text{CO}_2$ .

It may be observed that the third carbon of phenylalanine was not oxidized to  $\text{CO}_2$  by the tumor cells.

TABLE 7  
AMINO ACID OXIDATION BY GARDNER  
LYMPHOSARCOMA CELLS\*

Amino acid activity†	$\text{O}_2$ uptake ( $\mu\text{l.}$ )	Total counts added	Counts in $\text{BaCO}_3$	Per cent added counts in respiratory $\text{CO}_2$
None (control)	193			
Glycine-1- $\text{C}^{14}$ (3.71 $\mu\text{c}/\text{mg}$ )	186	47,100	220	0.5
$\text{DL}$ -Leucine-2- $\text{C}^{14}$ (1.55 $\mu\text{c}/\text{mg}$ )	178	38,900	453	1.2
$\text{DL}$ -Alanine-1- $\text{C}^{14}$ (3.6 $\mu\text{c}/\text{mg}$ )	193	41,150	5,383	13
$\text{DL}$ -Phenylalanine-3- $\text{C}^{14}$ (2.48 $\mu\text{c}/\text{mg}$ )	198	89,900	0	0

\* Final volume in main compartment of Warburg flasks, 1 ml.; 0.5 ml. cells, 0.1 ml. amino acid, 0.4 ml. KRP; 0.2 ml. 20 per cent KOH in center well. Incubated 3 hours at  $37^\circ$ , flushed with 100 per cent  $\text{O}_2$ . Each ml. packed cells diluted to 3 ml. to make cell suspension.

† Concentration in all instances was 1 mM/l.

## DISCUSSION

Evans and Bird, studying rabbit bone marrow cells (4), have reported that the addition of glucose to cell suspensions did not increase the net oxidation of the bone marrow cells. However, in the absence of glucose, the R.Q. was 0.83, and in the presence of glucose it was 0.91. Evans (5) reported similar data for the oxidation of pyruvate by the bone marrow cells. Moreover, isotope experiments by Barron *et al.* (1) have clearly demonstrated that bone marrow cells, incubated with acetate-1- $\text{C}^{14}$ , incorporated  $\text{C}^{14}$  into the protein of these cells in the form of dicarboxylic amino acids.

The situation is similar in the case of the Gardner lymphosarcoma. By isotope methods it was possible to demonstrate the oxidation of amino acids and of acetate to  $\text{CO}_2$ . The oxidation of the  $\beta$ -carbon of leucine and carboxyl-labeled acetate implies that the pathway is through the Krebs' cycle. The manometric demonstration of the oxidation of succinate provides further evidence for the tricarboxylic acid cycle pathway in this cell, as

does the demonstration of inhibitory effects on respiration and amino acid uptake by Krebs' cycle inhibitors. Furthermore, it has been shown that citric acid can be formed from acetic and oxalacetic acids by the tumor cells.

Recently, Weinhouse *et al.* (12, 13) have shown that radioactive palmitate and acetate are oxidized to  $\text{CO}_2$  by three other mouse tumors. These investigators have isolated radioactive quinidine citrate from flasks incubated with tumor tissue and labeled acetate or palmitate, and they have prepared purified enzyme preparations of the condensing enzyme, *cis*-aconitase, and isocitric dehydrogenase from the tumors which they studied. Pardee and co-workers (9) have also shown that two rat tumors oxidize acetate-1- $\text{C}^{14}$  to  $\text{CO}_2$ .

The sum total of this data makes it manifest that oxidation through the Krebs' cycle proceeds in tumor tissues. The experiments showing amino acid incorporation into the protein to be increased by succinate oxidation and the effects of respiratory inhibitors and anaerobiosis (6) on such uptake suggest that the energy from these oxidations is of great importance for protein synthesis in the tumor cells. It is possible that this energy is used for the formation of peptide bonds as a result of the generation of compounds containing high energy phosphate bonds. However, it is also possible that increased amino acid uptake into protein is attributable to an increase in the rate at which the labeled amino acid crosses the cell membrane. Christensen and Streicher (2) have shown that energy-yielding reactions are required for the concentration of amino acids within diaphragm cells. This process was inhibited by anoxia, cyanide, dinitrophenol, and arsenite.

## SUMMARY

It has been shown that the incorporation of four amino acids into the protein of the Gardner lymphosarcoma cells is markedly stimulated by the presence of glucose. Glycolytic inhibitors strongly suppressed the amino acid uptake.

The presence of succinate stimulated the respiration of the tumor cells, and, concomitant with this, a marked increase in the uptake of glycine and leucine and a slight increase in phenylalanine uptake into the cell protein were observed. Tricarboxylic acid cycle inhibitors suppressed oxidations and amino acid uptake.

Acetate-1- $\text{C}^{14}$  was partly oxidized to  $\text{CO}_2$  by the tumor cells and normal spleen cells. Some of the  $\text{C}^{14}$  from the acetate was found in the cell proteins. These processes and respiration were inhibited by fluoroacetate. Citrate was formed from acetate and oxalacetate by the tumor and normal spleen cells.

Alanine-1-C<sup>14</sup>, glycine-1-C<sup>14</sup>, and leucine-2-C<sup>14</sup> were also partly oxidized to CO<sub>2</sub> by the tumor cells. The implications of these results are discussed.

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# Tracer Studies on the Metabolism of the Gardner Lymphosarcoma

## III. The Rate of Radioactive Alanine and Glycine Uptake into the Protein of Lymphosarcoma Cells and Normal Spleen Cells\*

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### INTRODUCTION

It was reported by Zamecnik and associates (6, 7) that the rate of uptake of glycine-1-C<sup>14</sup> and alanine-1-C<sup>14</sup> into the protein of the *p*-dimethylaminoazobenzene-induced hepatoma slice is 7 times as great as that of the normal liver slice. This was assumed to imply a greater rate of peptide bond synthesis by the hepatoma. Since peptide bond synthesis is an indispensable part of the growth process, it was of interest to investigate whether or not an increased rate of amino acid uptake into tumor cells over normal cells is a constant accompaniment of neoplasia.

A study was therefore undertaken using the Gardner lymphosarcoma of the C3H mouse (2). The rate of uptake of C<sup>14</sup>-labeled glycine and alanine into the protein of cell suspensions of this tumor was compared to the uptake rate in cell suspensions derived from normal splenic tissue. The results are reported along with additional data on the uptake rate of cell suspensions from the spleens and lymph nodes of the tumor-bearing animals.

### METHODS

The methods employed in this study are essentially those reported in Paper I (1). The Dubnoff apparatus was used exclusively. Animals weighed between 20 and 25 gm. at the time that they were sacrificed.

\* Aided by research grants from the American Cancer Society, recommended by the Committee on Growth of the National Research Council, and the National Cancer Institute, Public Health Service.

† Abraham Rosenberg Research Fellow, 1949-50, Public Health Service Research Fellow of the National Cancer Institute, 1950-51. The work reported was taken from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry at the University of California.

Received for publication February 6, 1951.

*The tissues studied.*—The Gardner lymphosarcoma originated in the thymus of a C3H mouse after the subcutaneous implantation of an estrogen pellet (2). For this reason, the thymus lymphocytes of normal animals would be the tissue of choice for comparison with the tumor. The minute size of this organ in the mouse precluded its use.

The question of the relation of thymus lymphocytes to other lymphocytes has not been settled by histologists. However, on the basis of morphology, sensitivity to x-rays, toxins, and steroid hormones, it is generally believed that thymus lymphocytes are identical with lymphocytes found in other parts of the body (4). Immune sera against thymus lymphocytes cytolyze other lymphocytes (4).

Since the spleen represents the largest mass of lymphoid tissue in the body (4), suspensions of cells from the spleens of normal animals were used as the tissue of comparison. Experiments were also performed with suspensions derived from the spleens and lymph nodes of tumor-bearing animals. These, however, presumably contained malignant cells, since, when subcutaneously transplanted into normal mice, they grew and killed the animals. Normal spleens and lymph nodes were not transplantable.

*The cell suspension.*—The cell suspensions were made as previously reported (1). The suspensions of cells from the spleens of both the normal and tumor-bearing animals were centrifuged in a clinical centrifuge for 5 minutes. The bottom layer of erythrocytes was discarded. Thus, it was possible to eliminate many, but not all, the erythrocytes. The remaining packed cells were diluted with the KRPB solution (1), so that 0.5 ml. of the cell suspension contained about  $150 \times 10^6$  lymphocytes, as evidenced by cell counts. The cell suspensions from the other tissues contained an equivalent concentration of cells ( $\pm 20$  per cent). It must be

borne in mind, in this connection, that the specific activity (radioactivity per unit weight of protein) is measured.

## RESULTS

*Initial experiments.*—In contrast to the experiments with the rat hepatoma, the rate of glycine or alanine uptake was no greater in the lymphosarcoma cells than in the cells of the normal spleen, or the cells from the spleen and node of the tumor-bearing animal. The preliminary experiments, however, were conducted on tumor tissue which had grown in the mouse for 2 weeks. The possibility existed that after this period of growth, the tumor was no longer proliferating as actively as at 1 week of growth and that, consequently, its net protein turnover rate was not at a maximum. An experiment was therefore set up to investigate this point.

*Glycine uptake as a function of days after transplanting.*—The results for this experiment both in the presence and absence of glucose are shown in Chart 1. It may be seen that, in all cases, the specific activity of the normal spleen cells was used as the base line for comparison. The absolute value of the specific activity of these cells expressed as  $\mu\text{g C}^{14}/\text{gm protein}^1$  is given in the legend to Chart 1. It may be observed that the uptake varied only moderately from experiment to experiment.

Chart 1 shows that, in the presence of glucose, the glycine uptake for a 100-minute incubation was similar for all the tissues between 8 and 15 days after the tumor was transplanted. In the absence of glucose, however, the glycine uptake of the tumor was 60 per cent below that of the normal spleen cells. The node tissue was greatly enlarged after the tumor had grown for 13 days and undoubtedly contained a high proportion of tumor cells. It may be seen that its glycine uptake resembled that of the tumor in both the absence and presence of glucose.

In the presence of glucose, the specific activity of the protein of all the tissues was always considerably greater than in its absence.

*Alanine uptake as a function of days after transplanting.*—Chart 1 also shows data of experiments performed with alanine. Both in the absence and presence of glucose, the specific activity of the tumor was considerably below that of suspensions of the normal spleen and tumor-bearing animal spleen. The suspensions from the spleens of normal and tumor-bearing animals showed essentially the same activity. The lymph node seemed to represent a transition tissue, tending to resemble the

tumor to a greater extent as it became more enlarged.

The failure of the tumor tissue to equal the spleen tissue in alanine uptake in the presence of glucose may perhaps be attributed to the faster

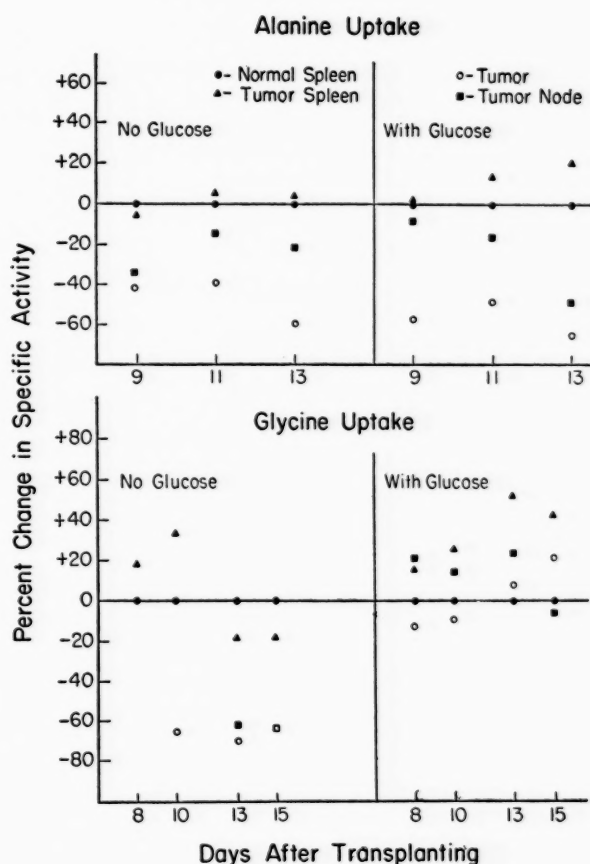


CHART 1.—Tumor node and tumor spleen represent the lymphocyte suspensions from the lymph nodes and spleens of tumor-bearing animals. Flasks were incubated 100 minutes at 38°, using KRPB solution. Gas phase: 95 per cent  $\text{O}_2$ –5 per cent  $\text{CO}_2$ . Glycine-2- $\text{C}^{14}$  ( $3.45 \mu\text{g}/\text{mg}$ ) concentration: 0.00125 M. DL-Alanine-1- $\text{C}^{14}$  ( $3.6 \mu\text{g}/\text{mg}$ ) concentration: 0.001 M. Glucose concentration: 0.0125 M in glycine flasks and 0.01 M in alanine flasks. Measured specific activities of normal spleen cells in  $\mu\text{g C}^{14}/\text{gm protein}$  were: (a) Glycine experiment, with glucose: 56, 71, 62, 62; no glucose: 37, 42, 45, 43 for days 8, 10, 13, and 15, respectively. (b) Alanine experiment, with glucose: 37, 25, 32; no glucose: 26, 12, 16 for days 9, 11, and 13, respectively.

rate of aerobic glycolysis of the tumor tissue. In the presence of glucose, pyruvate will be formed, and nonradioactive alanine, formed from this, may be expected to dilute the radioactive alanine already present. It has been possible to demonstrate directly that the addition of pyruvate to tumor cell suspensions containing no other substrate inhibits the uptake of alanine 45 per cent, while the uptake of glycine is not inhibited. This occurred despite an equal increase in respiration in the

<sup>1</sup> Here  $\text{C}^{14}$  merely designates radioactive carbon and is not intended to mean that the incorporation represents  $\text{C}^{14}$  solely.

flasks containing either amino acid plus pyruvate.

The data therefore did not suggest that the length of time that the tumor had grown in the mouse was a factor contributing to the failure of the tumor to exceed the normal lymphoid cells in amino acid uptake.

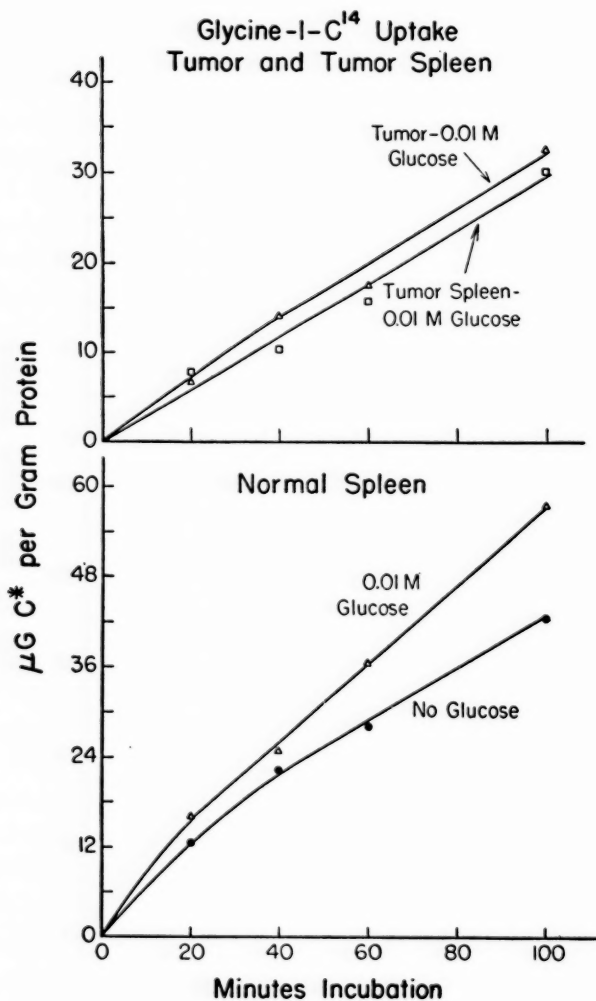


CHART 2.—Flasks contained 0.1 ml. of 0.01 M glycine-1-C<sup>14</sup> (3.71 μg/mg), 0.1 ml. of 0.1 M glucose, 0.3 ml. KRPB solution, and 0.5 ml. cell suspension in KRPB.

*Rate of glycine uptake 13 days after transplanting the tumors.*—In the experiments reported above, the cells were incubated for 100 minutes. It was desirable to determine the amino acid uptake as a function of incubation time in order to obtain a clearer picture of the synthetic potentialities of the cells. Tumor tissue was therefore taken 13 days after the tissue had been transplanted and compared with the other tissues.

It may be observed (Chart 2) that the glycine uptake of the tumor cells was linear in the presence of glucose, as was the uptake of glycine by the spleen cells from both the tumor-bearing and the normal animal. The rate of uptake of the tumor

was equal to that of the tumor animal spleen cells. In the absence of glucose, the rate of uptake of glycine by the normal spleen cells was less than that in the presence of glucose. However, glycine

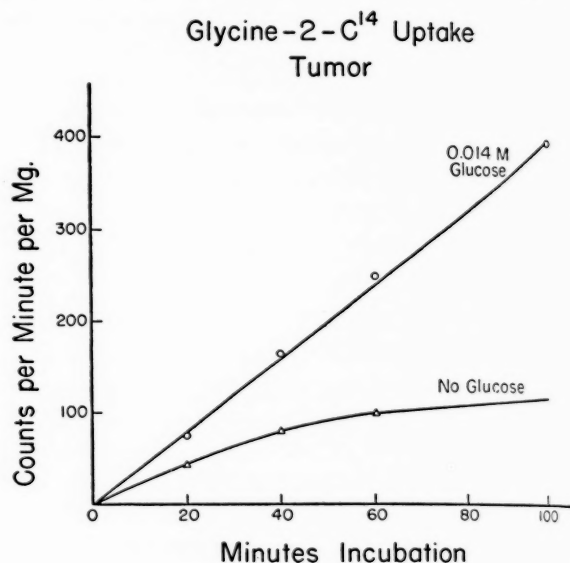


CHART 3.—Flasks contained 0.1 ml. of 0.02 M glycine-2-C<sup>14</sup>, 0.2 ml. of 0.11 M glucose, 0.2 ml. KRPB solution, and 0.3 ml. of tumor cell suspension.

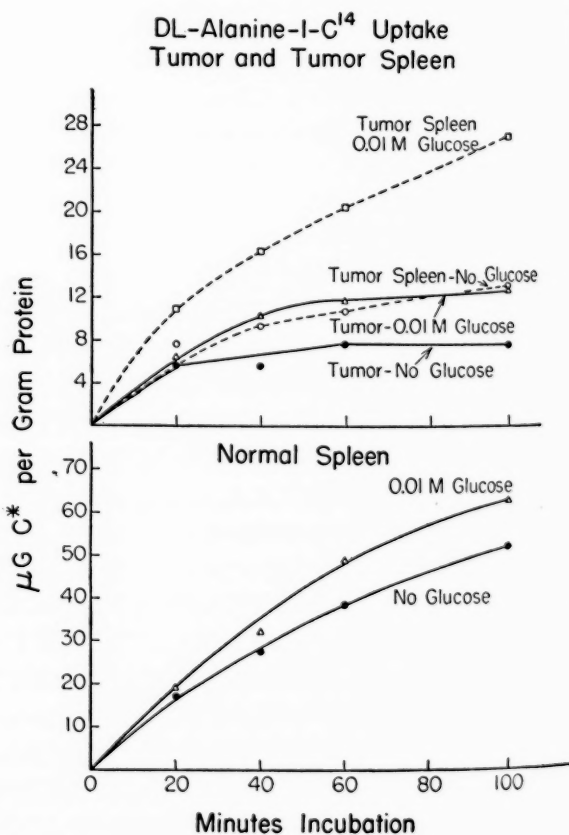


CHART 4.—Flasks contained 0.5 ml. cell suspension, 0.1 ml. 0.01 M DL-alanine-1-C<sup>14</sup> (3.6 μg/mg), 0.1 ml. of 0.1 M glucose, 0.3 ml. KRPB.

uptake persisted throughout the 100-minute incubation (Chart 2).

Chart 3 shows that in the absence of glucose, the rate of uptake of glycine was considerably less for the tumor cells than in the presence of glucose and that the process was no longer linear after the first 40 minutes.

*Rate of alanine uptake 13 days after transplanting.*—In Chart 4 are shown analogous data for alanine uptake. Here again it may be seen that the presence of glucose stimulated alanine uptake in all the tissues. The normal spleen cells showed a nearly linear uptake rate in the presence of glucose, and the rate was fairly well maintained in the absence of glucose. The rate of alanine uptake for the cells from the spleen of the tumor-bearing animal was nearly linear in the presence of glucose but plateaued after about 40 minutes in the absence of glucose. The tumor cells did not show linear alanine uptake either in the presence or absence of glucose, and in both the rate was considerably below the rate of the cells from the tumor animal spleen.

It would appear, therefore, that the rate of amino acid uptake of the lymphosarcoma cell is not greater than that of the normal spleen cell but is probably equal to it. Measured differences in the specific activity of the protein of these normal and tumor cells apparently depend upon the length of the incubation and the amount of time which has elapsed from the death of the animals to the beginning of an incubation. It has been shown in Paper I (Table 3) that at 38° C. the ability of the normal and tumor cells to incorporate glycine into their proteins is rapidly lost (1). The greater stimulation of uptake in the tumor with added glucose may be due to a lower level of energy-yielding reserve materials in the tumor. In this connection, it should be recalled that the Gardner lymphosarcoma is a transplanted tumor and is relatively avascular.

#### DISCUSSION

The contrast between the present result with the transplanted Gardner lymphosarcoma and that of Zamecnik and co-workers with the "spontaneous" hepatoma requires comment. It may be pointed out, first of all, that when labeled alanine or glycine is injected into intact animals, the rate of amino acid uptake by the hepatoma is not greater than that of the normal liver (3, 6). Also, in the intact animal, the rate of glycine uptake by the lymph nodes of normal animals appears to be less than that of the Gardner lymphosarcoma (5). However, in connection with these apparent contradictions, it is to be recalled that turnover studies in intact animals are complicated by the interactions of the tissues of the animal. By employing

an *in vitro* system, one eliminates this difficulty, although many others persist.

Amino acid uptake is assumed to provide a measure of peptide bond formation. It is to be emphasized, however, that as yet no conclusions can be drawn as to whether these peptide bonds are found in proteins, formed *de novo*, or are a result of an "exchange process" with pre-existing proteins. By postulating that the proportion of counts due to "exchange" reactions are the same in all the cell types studied in this paper, one would arrive at the interesting conclusion that the rate at which proteins are being synthesized in this tumor cell is not greater than in the normal cell.

In order to follow this thought a step further, a discussion based on Chart 5 is presented. Three kinds of normal cells are shown schematically. The first may be designated the "steady-state" cell. The squares within the circles are intended to represent the protein compartment of the cell. The shading emphasizes that the proteins are heterogeneous. It may be anticipated that all the proteins are not being synthesized and catabolized at the same rate. However, in time  $t$ , the average rate at which proteins are synthesized equals the average rate at which they are catabolized, so that although the spectrum of proteins within the cell may change, the net *amount* of protein remains the same. Secondly, there is the secretory-type cell. Endocrine gland cells which produce protein hormones, pancreatic acinar cells, or liver cells which release albumin into the serum exemplify this cell type. In time  $t$ , the average rate at which proteins are synthesized is not equal to the average rate at which they are catabolized. Thirdly, there is the mitotic cell such as the germplasm cell, the lymphoid cell, and the basal epidermal cell. Here again, in time  $t$ , the rates are unequal, so that a net formation of protein takes place.

Let us next make the simplest comparison between a normal cell and a tumor cell, that between a type I normal cell and a type III tumor cell. There has occurred an increase in the protein content of the tumor cell, as compared to the normal cell. This could have resulted from (a) an increased rate of synthesis with a slightly increased, equal, or a decreased rate of catabolism; (b) an equal rate of synthesis with a decreased rate of catabolism; or (c) a decreased rate of synthesis with a markedly decreased rate of catabolism.

Zamecnik and Stephenson (8) have recently reported data which tend to indicate that, with respect to the *p*-dimethylaminoazobenzene-induced hepatoma, there is an increased concentration of the protein part of catheptic enzymes but a decreased concentration of the activators of the same enzymes. These authors have also stated that the

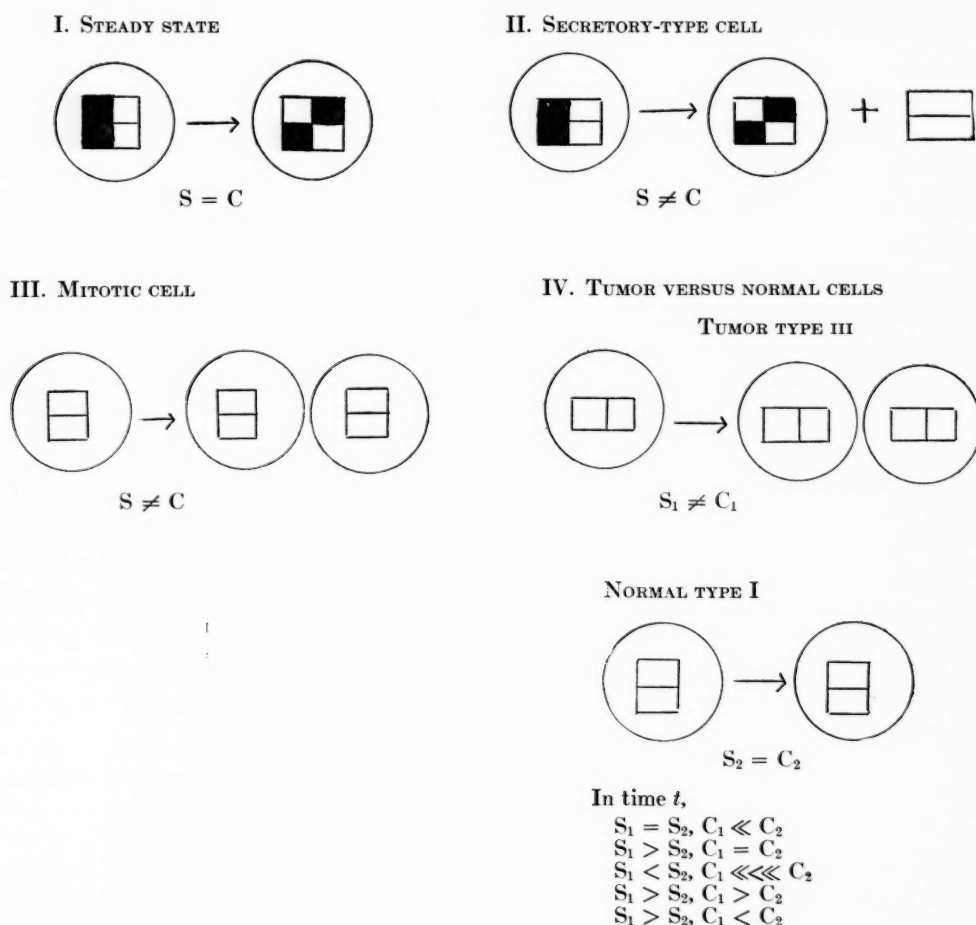


CHART 5.—Comparison of types of cells with respect to synthesis and catabolism of proteins

"rat hepatoma slice as compared with the normal rat liver slice, may have an increased concentrating mechanism for amino acids and an increased capacity to synthesize certain amino acids from their precursors" (6). It would therefore appear that many processes relating to protein metabolism are speeded up. Analogous data, however, are not yet available for the Gardner tumor.

The data reported in this paper, together with the fact that some human tumors are slowly growing and are fatal only after years of growth, emphasize that an increased rate of protein synthesis is not necessarily the basis for the continued and autonomous growth of all tumors.

#### SUMMARY

The rate of uptake of labeled alanine and glycine into lymphosarcoma cells, cells from the spleen and lymph nodes of tumor-bearing animals, and cells from the spleens of normal animals have been compared. The uptake of the tumor cells was not greater than that of the other cells under several experimental conditions. The implications of this are discussed.

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# Experimental Studies on the Etiology of Hodgkin's Disease\*

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For many decades Hodgkin's disease has been looked upon as a type of malignancy that possesses many characteristics which seem to set it apart from most of the other cancerous processes. Many of these characteristics have been ones that suggest an inflammatory background to the tumor, such as the microscopic granulomatous appearance, the fever, the cyclic exacerbations and remissions of the disease, and the partial anergic state. In fact, most of the early workers interested in Hodgkin's disease considered it to be in some way related to known infections.

Excellent and complete recent reviews of Hodgkin's disease are available (Hoster *et al.* [11], Jackson and Parker [12], and Wallhauser [20]) that present the evidence for and against the virus and nonvirus concepts of the etiology of Hodgkin's disease. A presentation of that same data would serve no purpose here.

During the past decade the technic of using fertile chicken eggs for the study of virus diseases has come into its own. Although used in the study of neoplasms as early as 1913 by Murphy (14) and re-used for experiments with viruses by Goodpasture *et al.* (9) in 1932, the thorough study of methods by many workers was required before reproducible observations could be made.

This new medium of study, which has proved itself so adaptable to virus research, presented a new approach to the relationship of Hodgkin's disease to any possible virus-like agent. Bostick (3) commenced a preliminary survey of Hodgkin's disease by the systematic use of the various methods of chicken embryo passage. By recording the effect of Seitz-filtered serially passed Hodgkin's disease extract in chicken eggs, he noted a slight but statistically greater mortality of embryos inoculated with Hodgkin's disease material than with carefully prepared control tissue extracts.

Amniotic fluid, harvested after at least from 4 to 15 serial passages, was employed in all observations reported here.

With this provocative initial success, all the pos-

\*This investigation was supported in part by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service.

Received for publication February 6, 1951.

sible variations in virus and chicken egg experiments were methodically surveyed. The technics studied are listed below under "Methods." The majority of the methods failed to give any evidence of a difference between the Hodgkin's disease and the control material. However, since all methods were explored at considerable expense of time, animals, and material, it is of value to indicate in this survey all the avenues of investigation used and the results obtained. It is apparent that certain methods should be abandoned as apparently fruitless avenues of research, and others are being pursued with increasing promise.

## METHODS AND RESULTS

The first studies were directed toward demonstrating a lethal factor by its direct effect on the embryo or its membranes. Many of the known viruses which can be successfully cultivated in embryonated eggs cause the death of the embryos in a fairly uniform interval of time. Lesions which sometimes develop on the chorioallantois have provided a crude method of determining the amount of certain viruses, and in some viruses specialized tissues of the embryo may show pathologic changes characteristic of the infectious process.

The results of the mortality studies have been reported in detail elsewhere (Bostick [3]). Fresh Hodgkin's disease lymph nodes were ground under sterile conditions, diluted 1:10 in 0.85 per cent NaCl, Seitz-filtered, and inoculated in 0.02-ml. amounts into the amniotic sacs of fertile chicken eggs which had been incubated for 7 days at 38.5° C. Control material consisting mainly of carcinomatous lymph node extracts was treated in an identical manner. Deaths occurring during the 24-hour period immediately following inoculation were considered nonspecific. Mortalities were checked for the next 10-day period. Amniotic fluid was harvested from dead embryos and stored at 4° C. for further transfer. Bacteriologic studies were made at all stages, and upon any secondary contamination the material was Seitz-filtered before subsequent passage. Average mortalities were calculated for each of two groups, based on a total of over 2,700 eggs. In the Hodgkin's disease group A the mortality was 18 per cent higher than in the

control, and in the Hodgkin's disease group B the mortality was 11 per cent greater than in the controls. Both of those differences were shown to be statistically significant ( $P = 0.001$  and  $P = 0.005$ , respectively). The greatest difference in mortality was shown to occur between the fifth and sixth days.

No specific gross or microscopic lesions could be demonstrated in live or dead embryos. Hematoxylin and eosin stains were used for the general histologic studies, Machiavelli stains for inclusion body studies. Tissues examined included the chorioallantoic and amniotic membranes, lung, and liver from normal control and experimental embryos.

If the effect of any agent under investigation were more debilitating than lethal to the embryo, a difference in weight between control and Hodgkin's disease series might be demonstrable.

TABLE 1

COMPARISON OF EMBRYO WEIGHT IN HODGKIN'S DISEASE AND CONTROL INJECTED SERIES

	HODGKIN'S DISEASE		CONTROL	
	No. of embryos	Av. wt. (gm.)	No. of embryos	Av. wt. (gm.)
Dead at 6 days	11	4.71	10	4.82
Alive at 6 days	36	5.44	13	5.31
Alive at 9 days	35	10.50	16	11.21

Weights were determined at 6 or 9 days following inoculation (i.e., 13- or 16-day embryos). Dead embryos were weighed as soon as possible after death, live embryos after 2 hours of chilling at 4° C. The difference was so slight as to be insignificant (Table 1).

The protein content of the inoculated eggs was studied and compared. The amount of protein in the amniotic fluid from 12-day live control and experimental eggs was determined. Determinations were based on readings in a Klett-Summerson colorimeter 10 minutes after addition of sulfosalicylic acid. Each fluid was tested separately or as a pool of 5-9 eggs obtained from the same passage. No constant difference between control and Hodgkin's disease amniotic fluids was noted.

Because patients with Hodgkin's disease may show a debilitating effect which often seems to be much greater than the actual extent of the tumor involvement, the possibility existed of a toxic effect of the Hodgkin's disease agent similar to that present in LGV (Rake and Jones [16]). Yolk sacs from routinely inoculated control and Hodgkin's disease eggs were harvested after a 6-day incubation period. Suspensions were prepared by grinding with sterile sand or by shaking at 187 oscillations per minute for 1 hour; 0.5-ml. amounts were injected intravenously into series of mice which

were examined for symptoms after 2 and 4 hours and 1, 2, 3, and 4 days. At the end of 4 days all mice were still alive and healthy.

#### HEMAGGLUTINATION AND RELATED TECHNIQUES

Hemagglutination of erythrocytes by viral agents and the neutralization of the causative hemagglutinins by immune serum were first demonstrated by Hirst (10) in his work with influenza. Since that time many other viruses have shown this property when grown in fertile chicken eggs, either in the extra-embryonic fluids or in a suspension of the ground fetal membranes. Further-

TABLE 2

SURVEY OF POSSIBLE HEMAGGLUTINATION EFFECTS OF HODGKIN'S DISEASE AMNIOTIC FLUID ON THE ERYTHROCYTES OF VARIOUS ANIMALS

SOURCE OF ERYTHROCYTES	AMNIOTIC FLUID		GROUND MEMBRANES SUSPENDED IN AMNIOTIC FLUID	
	No. tested	Results	No. tested	Results
Human (normal)	6	negative	1	negative
Human (Hodgkin's disease)	6	negative		
Chicken (normal)	6	negative	2	negative
Chicken (injected with Hodgkin's disease)	1	negative		
Rat (normal)	3	negative	1	negative
Guinea pig (normal)	1	negative	1	negative
Rabbit (normal)	1	negative		
Mouse (normal)	1	negative	1	negative
Dog (normal)	1	negative	1	negative
Sheep (normal)	1	negative	1	negative
Goat (normal)	1	negative		
Goose (normal)	1	negative		
Monkey (normal)	1	negative	1	negative
Turkey (normal)	1	negative		
Hamster (normal)	1	negative	1	negative
Cow (normal)			1	negative
Hog (normal)			1	negative

more, it has been demonstrated (Burnet *et al.* [5]) that red blood cells "sensitized" by one virus may be agglutinated by certain other viruses which previously had no agglutinative effect on erythrocytes. Such sensitized cells are usually agglutinated by most normal sera in only low dilutions and by related antisera in high dilutions.

Direct hemagglutination tests were set up with amniotic fluid in serial solutions, beginning with  $\frac{1}{10}$  in 0.2-ml. volumes. Equal parts of 0.85 per cent NaCl and of 0.75 per cent RBC suspension were added, with shaking. Readings were made after 1, 2, or 3 hours, varying according to the erythrocyte donor species. The supernates of centrifuged ground membrane suspensions were handled similarly. In the same manner series were set up in which Hodgkin's disease patients' sera were added to normal human or chicken erythrocytes or those from Hodgkin's disease-injected chickens. None of these gave any indication of hemagglutination (Table 2).

With the hemagglutination by Hodgkin's disease amniotic fluid material apparently not demonstrable, attention was then directed to a study of the effect of human sera, either Hodgkin's disease or control, on the hemagglutinative capacities of other viruses, particularly mumps, vaccinia, NDV, influenza PR8, and Lee. The sera used were from normal and Hodgkin's disease humans and normal and Hodgkin's disease-injected chickens.

To determine any sensitization or stabilizing effect that the Hodgkin's disease amniotic fluid might have on erythrocyte hemagglutinative properties, a series of tests was set up in which erythrocytes were added to undiluted amniotic fluid containing one of the following agents: PR8, Lee, NDV, mumps, vaccinia, or Hodgkin's disease. These erythrocytes were then incubated at 37° C. for 2 hours with frequent shaking (to elute the vi-

TABLE 3  
NEUTRALIZATION OF HEMAGGLUTINATION BY SERA

VIRUS	CHICKEN ERYTHROCYTES		HUMAN ERYTHROCYTES	
	Chicken sera	Human sera	Chicken sera	Human sera
	No. tested	Results	No. tested	Results
Newcastle disease virus (NDV)			3	Neg.
			4	?
Mumps virus (MEV)	2	Neg.	2	?
Influenza (PR8)	2	Neg.	1	Neg.
Influenza (Lee)				2
Vaccinia	2	Neg.	1	Neg.

TABLE 4  
AGGLUTINATION OF "SENSITIZED" ERYTHROCYTES BY HODGKIN'S DISEASE FLUIDS

	HUMAN ERYTHROCYTES				CHICKEN ERYTHROCYTES			
	Amniotic fluid		Hodgkin's disease		Amniotic fluid		Hodgkin's disease serum	
	Tests	Results	Tests	Results	Tests	Results	Human	Chicken
NDV	1	Neg.			1	Neg.	1	Neg.
Vaccinia			2	Neg.				1
Mumps	1	Neg.	1	Neg.				1
PR8	1	Neg.	1	Neg.				
Lee	1	Neg.	1	Neg.				

TABLE 5  
EFFECT OF HODGKIN'S DISEASE AMNIOTIC FLUID "SENSITIZATION" OF ERYTHROCYTES

	HUMAN ERYTHROCYTES "SENSITIZED"		CHICKEN ERYTHROCYTES "SENSITIZED"	
	BY HODGKIN'S DISEASE		BY HODGKIN'S DISEASE	
	Tests	Results	Tests	Results
Hodgkin's disease human serum	1	Neg.	6	Neg.
	1	?		
Hodgkin's disease-injected chicken serum			1	Neg.
Infectious mononucleosis human serum	1	Neg.	2	Neg.
	1		1	?

The sera were usually used in a raw state, but in some instances were inactivated at 56° C. for 30 minutes. Either chicken or human erythrocytes were used. Doubling serial dilutions of serum plus 4 hemagglutinative units of one of the above-named viruses, or doubling dilutions of that virus plus  $\frac{1}{10}$  serum, were incubated at 37° C. for 45 minutes followed by room temperature for 15 minutes. (Other variations of time and temperature showed no advantage over the standard procedure.) Following this initial incubation period, 0.75 per cent erythrocytes were added and allowed to settle for 1 or 2 hours, depending upon the species of erythrocytes used, before the degree of hemagglutination was recorded (Table 3).

rus if possible), centrifuged, and the resultant "sensitized" cells resuspended in normal saline. This "sensitized" cell suspension was then added to serial dilutions of Hodgkin's disease amniotic fluid or normal or Hodgkin's disease serum, and readings were made for the presence or absence of hemagglutinations after 1 or 2 hours at room temperature (Table 4). Hodgkin's disease-sensitized erythrocytes were also checked against serum from infectious mononucleosis patients (Table 5). As shown in these tables, no differences could be demonstrated between control and Hodgkin's disease material.

*Serologic studies.*—A search was made for the existence of specific antibody formation by means

of some of the standard procedures of flocculation, precipitin or complement fixation. The last, particularly, has been used to a great extent as a diagnostic aid in viral and rickettsial diseases. Preliminary surveys have been conducted in an attempt to demonstrate any obvious antigenic reaction by Hodgkin's disease amniotic fluids.

In the slide flocculation test a procedure was used which was similar to that of the Kline-diagnostic test for syphilis. One per cent cholesterolized alcohol (0.5 ml.) was added to an equal amount of 0.85 per cent saline. This suspension was shaken vigorously for 1 minute, 0.8 ml. of pooled Hodgkin's disease amniotic fluid was added and mixed, and 0.5 ml. (0.85 per cent) saline added to bring the final emulsion to an optimum density. This was reshaken less vigorously for 1 minute, incubated at 53° C. for 15 minutes, added to 0.05 ml. serum on ringed slides in amounts of approximately 0.008 ml. followed by 4 minutes of rotation. When checked with this antigen, raw sera gave no reaction at all; inactivated sera showed a nonspecific clumping of particles.

Precipitin studies consisted of the careful laying of 0.1 cc. pooled Hodgkin's disease amniotic fluid (1:5 dil.) over 0.1 cc. serum (undiluted or 1:2), in 95 × 5 mm. tubes. Sera used were from control and Hodgkin's disease-injected chickens, Hodgkin's disease-injected and normal humans. Readings were made after 20-, 60-, and 120-minute incubation at 37° C. No reaction could be noted by macroscopic observation in the series of chicken sera nor any difference between Hodgkin's disease and control human sera.

Complement fixation tests have been performed and are being thoroughly investigated. The technique of Bengtson (1) has been followed, and the reagents have consisted of commercial lyophilized complement and amboceptor, fresh sheep erythrocytes, and inactivated human sera. When collected, these data will be presented in a later article.

*Sensitivity (cutaneous).*—Intradermal sensitivity tests have become fairly well established as diagnostic procedures, particularly the Frei test for LGV, but also with influenza (Beveridge and Burnet [2]), vaccinia (Smith [18]), mumps (Enders *et al.* [6]) and herpes simplex (Rose and Malloy [17]). In these various tests a wide range of reactions was noted, with from 54 per cent to 93 per cent of patients with known histories of infection giving positive results.

Amniotic fluid from Hodgkin's disease and control series of embryos 8–12 days old were carefully harvested, pooled, checked for bacterial contamination, and the protein level was determined to be less than 200 mg. per cent. The material was in-

activated by heating to 70° C. for 15 minutes. In some instances this was followed by 4 minutes' ultraviolet radiation at 15 inches. The addition of 1:10,000 Merthiolate was discontinued after several mercury-sensitive individuals were encountered. Injections of 0.1-cc. amounts of several such antigens were administered intradermally into the forearms of a group of Hodgkin's disease and non-Hodgkin's disease patients ranging in age from 1 to 79. The results demonstrated only a variable and slightly increased sensitivity of Hodgkin's disease and of some control patients to Hodgkin's disease antigen. This was too irregular in frequency and too slight a degree of response to represent a promising avenue of study.

*Animal inoculations.*—Repeated attempts have been made in the past to inoculate various animals with Hodgkin's disease lymph node extract. In the present survey it was felt that further study was indicated with the use of Hodgkin's disease amniotic fluid. On the assumption that the agent might have become chicken-adapted through repeated egg passage and, in order to rule out nonspecific foreign protein effects, most of the experiments were done with chickens. Two separate series were set up. The first consisted of six adult chickens, three inoculated with Hodgkin's amniotic fluid and three with control amniotic fluid. Intramuscular inoculations were made over a period of approximately 2 months, the dosage increasing from 0.5 to 1.5 cc. The Hodgkin's disease material was originally from four patients, the control from five. At autopsy, 2 or 3 months after the first injection, the tissues were carefully examined grossly and microscopically, and, when possible, blood was obtained for serologic study. A second experiment, consisting of only one chicken in each group, was set up at a later date. No distinction could be made in either series with regard to tissue changes or to serologic reactions in various hemagglutination tests referred to above.

Attempts to demonstrate development of intradermal sensitivity were made during the study of the effects of Hodgkin's disease inoculation. Chickens, guinea pigs, rabbits, rats, hamsters, and monkeys were used. Hodgkin's disease and control material, consisting of human lymph node cell-free extract, was injected into separate series of animals, the volume varying from 0.1 cc. to 0.3 cc. according to the size of the animal, and repeated 4–6 times. After an interval of 10 days, 0.1-cc. amounts of amniotic fluid from appropriate Hodgkin's disease and control cases were injected intradermally. The intensity of the reaction and actual diameter of any wheal were carefully determined after 24 hours. In some instances the guinea pigs showed a

nonspecific reaction, and results were inconclusive in regard to the rats. All other species showed no response whatever to the Hodgkin's disease material.

Among other animal experimental work, one series of seven experimental and seven control young adult mice was inoculated intracerebrally. They developed no signs and symptoms and upon post mortem examination showed no histologic changes. Attempts to produce corneal lesions (Goodpasture [8]) in either rabbits or guinea pigs were also unsuccessful.

*Interference.*—It has been established by many workers (Price [15], Vilches and Hirst [19], Ginsberg and Horsfall [7]) that in certain circumstances the presence of one virus will interfere with the growth of a subsequently inoculated one. In some instances a definite order of inoculation must be followed, while others inhibit one another regardless of the order of inoculation. The time interval between inoculations is often very important and varies with each combination of viruses.

Following preliminary surveys in which Hodgkin's disease was used as the first inoculum and various known viruses, including PR8, Lee, NDV, mumps, and LGV as the second, a large series have been run in which Hodgkin's disease fluid from 12-day live, fertile chicken eggs was inoculated into 7-day embryos (amniotic sac) followed 3 days later by Lee virus (Bostick [4]). The amniotic fluid was harvested 18 hours later and titered for the presence of Lee virus by the determination of its hemagglutinins, with human erythrocytes. Control materials tested have included normal amniotic fluid, as well as control material from non-Hodgkin's disease malignancies. Both Hodgkin's disease and control materials were carried through at least four egg passages before being tested. All materials were stored in a deep-freeze ( $-20^{\circ}\text{C}.$ ) refrigerator between passages. The results are shown in Table 6 below. The degree of interference

eggs inoculated originally with lymph nodes ground and pooled from several Hodgkin's disease patients, but by those from individual Hodgkin's disease patients. This capacity to interfere with the growth of the viruses will provide a greatly simplified tool for the study of the Hodgkin's disease-isolated agent.

## DISCUSSION

The application of a new but established technique to an old problem may result in the discovery of important facts which go far toward the solution of the problem. Hemagglutination phenomena were studied from many aspects. The Hodgkin's disease amniotic fluid was not hemagglutinative for any of many types of erythrocytes. It was also incapable of altering or "sensitizing" erythrocytes so that they would possess aberrant hemagglutination responses to most of the known hemagglutinative viruses. The Hodgkin's disease factor could not, therefore, be detected by these methods.

The Hodgkin's disease amniotic fluid was tested for an immediate toxic effect in various ways. Intravenous inoculations into adult mice failed to demonstrate any immediate or delayed toxic or infective effect of the material. Such animals are very sensitive to certain known viruses when administered in that manner.

In the chicken embryo, in addition to the mildly toxic effect of the Hodgkin's disease amniotic fluid, there is a tendency for an increase in the protein level of the amniotic fluid. However, this was of questionable significance and was not reflected in any abnormal change of weights of these embryos.

Most of the standard serological methods have failed to demonstrate any specific characteristic in the Hodgkin's disease amniotic fluid. Precipitin tests were negative, as were flocculation tests modeled after the Kline technic. Complement fixation methods were the only ones which showed some trend of specificity, and further data are being collected on them.

In agreement with the observations of many preceding investigators, it was impossible to cause any changes in many kinds of experimental animals upon inoculation with Hodgkin's disease lymph nodes emulsion or Hodgkin's disease amniotic fluid material. No intradermal sensitivity to this material could be detected in the injected animals.

The best approach for further exploration is the study of virus interference phenomena. Since the Hodgkin's disease agent itself possessed no easily identifiable effect on the chicken egg, its ability to interfere with the growth of a virus known to possess characteristic properties was investigated.

TABLE 6

INTERFERENCE WITH INFLUENZA VIRUS (LEE) BY  
HODGKIN'S DISEASE AMNIOTIC FLUID

	Interference by Hodgkin's disease	No inter- ference	Reversal
No. of tests	18	8	4
Percentage	60	27	13

of the growth of the Lee virus by the Hodgkin's disease agent was variable. Sometimes there was complete absence of the virus, as demonstrated by both hemagglutination and infectivity tests. On other occasions it was manifest by a decreased hemagglutination titer. This phenomenon was demonstrable not only by the amniotic fluids from

Several viruses were studied according to their ability to grow in fertile chicken eggs previously inoculated with the Hodgkin's disease agent. The growth of Lee influenza virus was noted to be susceptible to interference by the Hodgkin's disease material; initial inoculation depressed or completely interfered with the ability of the Lee virus to grow. Its growth was judged by hemagglutination titer methods and, on occasion, by egg-infectivity titers. Careful parallel controls were run at all stages. This reproducible and quite rapid test for the presence of the Hodgkin's disease amniotic fluid material will greatly aid in the further study of Hodgkin's disease.

The Hodgkin's disease amniotic fluid harvested following numerous serial egg passages is most satisfactory for study by certain physical-chemical procedures. This material is now being studied by electron microscope, ultracentrifuge, and differential precipitation techniques.

#### SUMMARY

The possible infectious characteristics of Hodgkin's disease have been re-examined by the previously unused technic of serial passage of the Hodgkin's disease lymph node extracts in embryonated chicken eggs. A slight lethal effect in the embryos was noted. The harvested amniotic fluid was shown to possess virus growth interference activities against Lee influenza virus grown in chicken eggs.

This harvested amniotic fluid was examined for other properties. No hemagglutination tendencies were encountered when tested against erythrocytes from many animals. No sensitization effects on erythrocytes later exposed to known hemagglutination viruses were noted. The amniotic fluid had no untoward effect upon animal inoculation via many routes. Precipitin and flocculation tests were uninformative, and complement fixation procedures showed some trends that deserve further study.

The amniotic fluid harvested after numerous serial passages from Hodgkin's disease-inoculated embryos has been shown to possess certain filterable, transferable, and virus-like properties. This material should make possible extensive studies by serological, chemical, and physical methods.

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# The Metabolic Degradation in the Mouse of 1,2,5,6-Dibenzanthracene-9,10-C<sup>14</sup>

## II. 5-Hydroxy-1,2-naphthalic Acid, a New Metabolite\*†

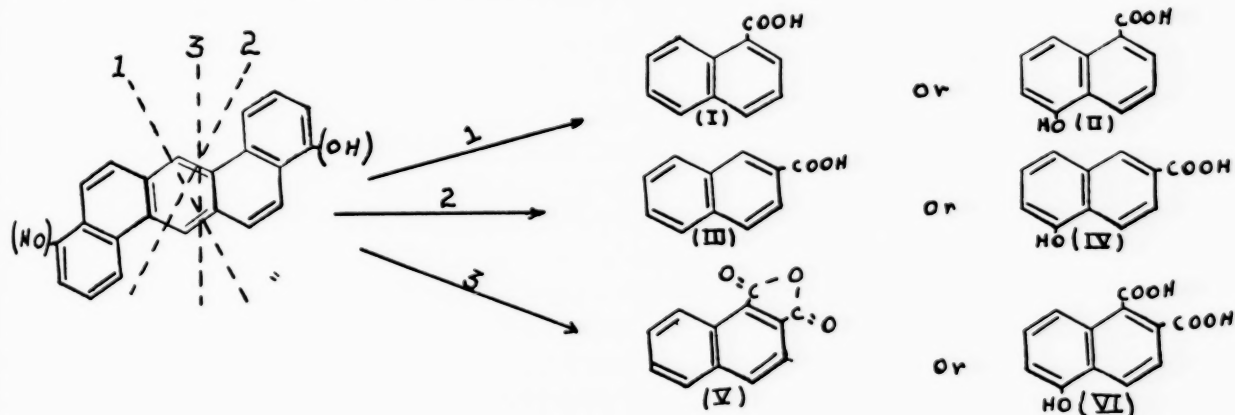
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(McArdle Memorial Laboratory, the Medical School, University of Wisconsin, Madison 6, Wis.)

The only metabolite of 1,2,5,6-dibenzanthracene in the mouse that has been identified with certainty was shown by Dobriner *et al.* (6) and by Cason and Fieser (4) to be 4',8'-dihydroxy-1,2,5,6-dibenzanthracene. Other workers (12, 13) using spectroscopic methods of analysis failed to obtain quantitative recoveries of the administered dose or to recognize metabolites other than the dihydroxy derivative. This was due to the fact that the characteristic ultraviolet absorption spectrum of dibenzanthracene is given only by the intact

It was further shown<sup>1</sup> that about 8 per cent of the acidic radioactivity is derived from carboxyls originating from carbons 9 or 10, proving that oxidation and cleavage of the central ring occur.

To investigate this problem further, the carrier technic was selected, which has unique advantages for this type of work. With it one can accurately identify substances on the microgram scale, and these can be isolated, with a minimum of effort, from large quantities of impurities. The procedure involves the addition of a known nonradioactive



pentacyclic aromatic ring, and any metabolite resulting from cleavage or alteration of the aromatic character of the hydrocarbon could not be identified unequivocally by this analytical method. Another technic for these studies was made available following the synthesis of 1,2,5,6-dibenzanthracene-9,10-C<sup>14</sup>(9); and by measuring the radioactivity of tissues and their fractions following the administration of the labeled carcinogen to mice, Heidelberg *et al.* (10, 11) were able to obtain quantitative distributions of radioactivity and to demonstrate the production of acidic metabolites.

\* An abstract of this report appeared in *Cancer Research*, 10:223, 1950.

† This work was supported in part by a grant from the Wisconsin Section of the American Cancer Society.

Received for publication February 16, 1951.

compound (the carrier) to a radioactive solution. If the carrier is chemically identical with a radioactive compound present in the solution, the two will mix. The radioactive material will then be present in and inseparable from the carrier, when it is recovered.

The following nonradioactive compounds, which could result from cleavage by the three ways indicated, have been added to an acidic radioactive fraction obtained from mouse feces:<sup>1</sup> 1-naphthoic acid (I), 5-hydroxy-1-naphthoic acid (II), 2-naphthoic acid (III), 5-hydroxy-2-naphthoic acid (IV), and 1,2-naphthalic anhydride (V). Other compounds which might be produced by further oxidative cleavage of the terminal rings have also

<sup>1</sup> C. Heidelberg, unpublished data, University of California.

been tested: benzoic acid, phthalic acid, benzene-1,2,3-tricarboxylic acid, and benzene-1,2,3,4-tetracarboxylic acid. None of these compounds carried radioactivity after purification, and hence they are not metabolites.

The only other possibility likely to result from

were fed a whole grain ration and tap water *ad libitum*. The mice were kept in metabolism cages, and feces were collected. No attempt was made to trap respired carbon dioxide, since it has been shown (10) that insignificant amounts of radioactivity are eliminated by this route. In all cases,

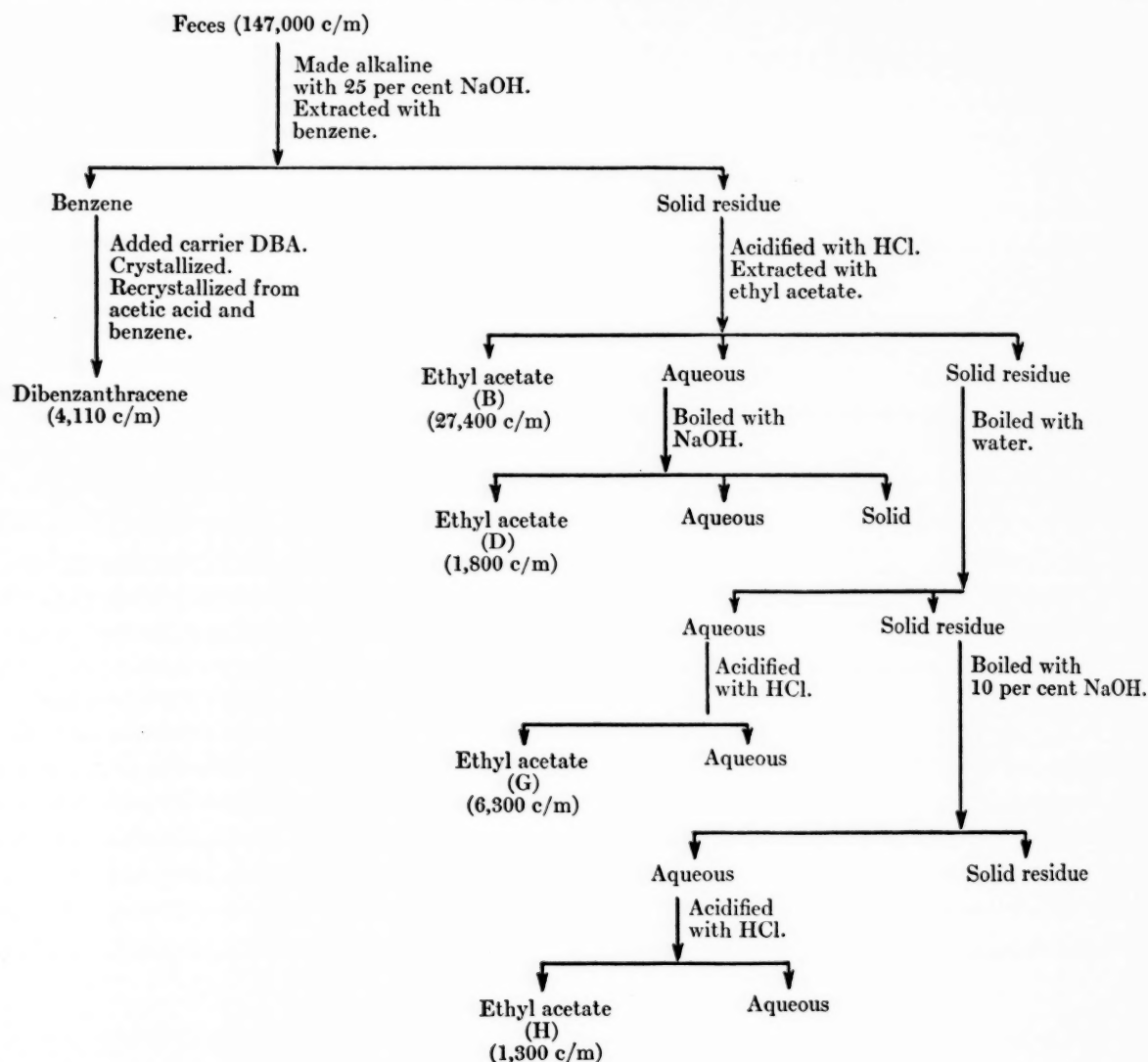


CHART 1.—Fractionation of feces

cleavage in the 9 and 10 positions is 5-hydroxy-1, 2-naphthalic acid (VI), which was not available at the time of the earlier experiments. This compound has now been synthesized and kindly furnished to us by Prof. W. G. Dauben (5), University of California. The following experiments prove that it is indeed a metabolite.

#### METHODS

*Treatment of animals.*—In all experiments the animals used were adult female albino mice.<sup>2</sup> They

<sup>2</sup> "Rockland" strain obtained from Arthur Sutter, Springfield, Mo.

except the skin-painting experiments, the radioactive hydrocarbon was administered intravenously as an aqueous colloid, prepared as described previously (10).

*Treatment of feces.*—Feces were dried at 60° C. in a vacuum oven, ground to a fine powder which would pass through a 0.5-mm. mesh wire screen, and the radioactivity was determined after combustion of an aliquot. Chemical fractionation was then carried out according to Chart 1.

*Treatment of liver.*—Mice were killed with ether, and the liver was removed from each mouse and

homogenized immediately in a Waring Blender with 15 ml. of 0.1 N sodium hydroxide. The blender was washed with 5 ml. water, and the washings were added to the homogenate. An aliquot of the homogenate was then combusted, and its radioactivity was determined. The remainder was placed on a steam bath, and the excess water was removed in a current of air. The last traces of water were removed azeotropically with benzene,

samples were counted in internal-sample counters, "Q-gas counters," used in conjunction with Berkeley Decimal, G-M Scalers, Model 1000. Counting of samples was continued long enough to give an accuracy of at least 10 per cent.

#### PROPERTIES OF 5-HYDROXY-1,2-NAPHTHALIC ACID

In the paper (5) describing the synthesis of 5-hydroxy-1,2-naphthalic anhydride, no mention is

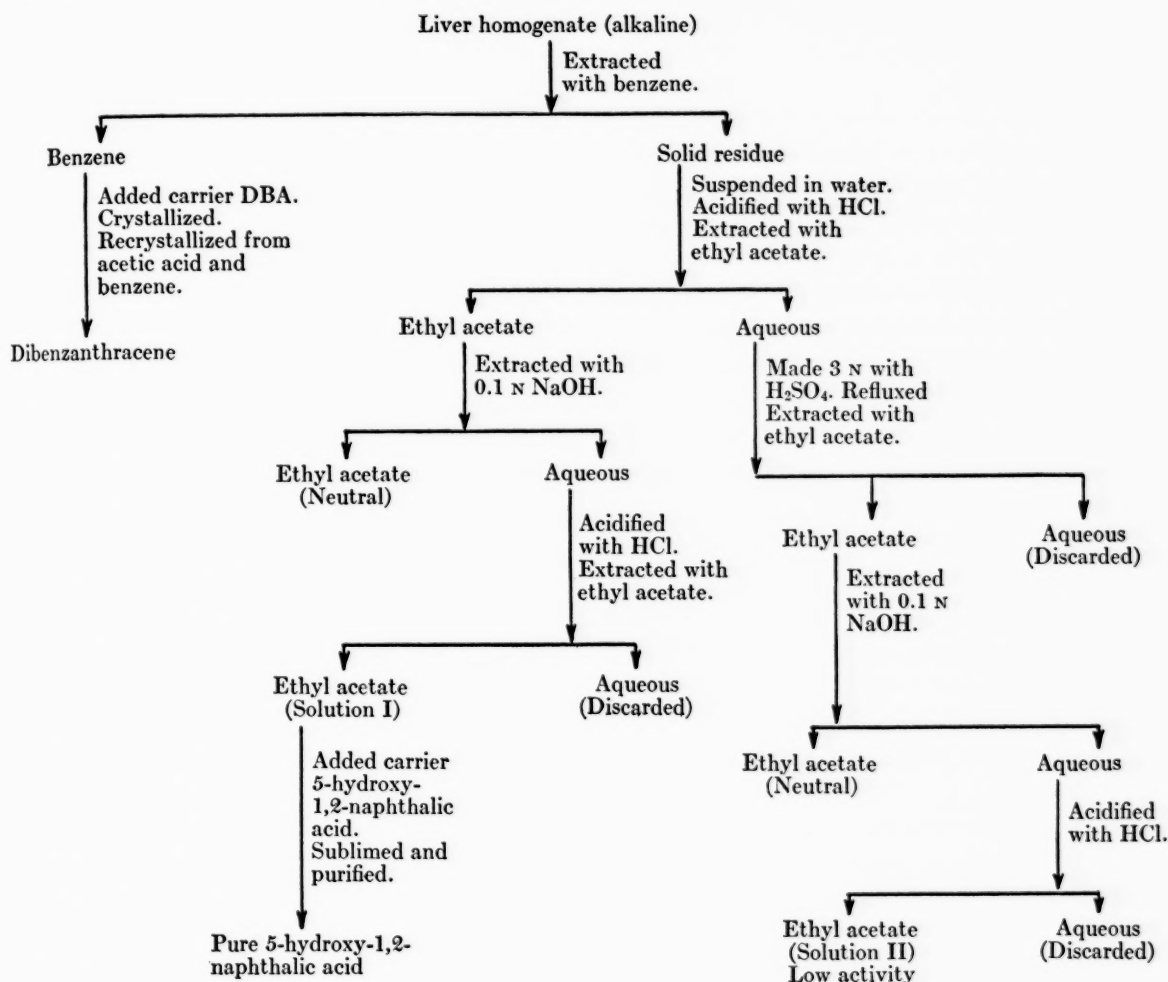


CHART 2.—Fractionation of liver

and extraction of the liver was carried out according to Chart 2.

Combustion of both solid and liquid samples was performed with the oxidizing reagent of Van Slyke and Folch (17). The carbon dioxide evolved was trapped in sodium hydroxide and precipitated with barium chloride. The barium carbonate was plated onto aluminum discs by evaporating a slurry of the solid in alcohol (3). Solutions of 5-hydroxy-1,2-naphthalic anhydride were evaporated onto aluminum discs by the direct plate method (3) without combustion. All radioactive

made of 5-hydroxy-1,2-naphthalic acid. We have shown that both the anhydride and the acid are stable compounds and are interconvertible. In this laboratory, the method of synthesis used by Dauben and Tanabe (5) gave a flesh-colored product after crystallization from water, m.p. 265°–266° (uncorrected).

*Analysis:* Calcd. for  $C_{12}H_{10}O_6$ : C, 57.60; H, 4.03. Found: C, 57.57; H, 4.36.

This empirical formula corresponds to 1 molecule of 5-hydroxy-1,2-naphthalic acid with 1 molecule

of water of hydration. When this compound was sublimed at a vacuum of 0.8 mm. and 150°, it gave a yellow compound having the same melting point as the acid. Analysis has shown this substance to be 5-hydroxy-1,2-naphthalic anhydride.

*Analysis:* Calcd. for  $C_{12}H_6O_4$ : C, 67.37; H, 2.82.  
Found: C, 67.32; H, 3.15.

The anhydride can be recrystallized from benzene and acetic acid, but, on recrystallization from water, it is reconverted to 5-hydroxy-1,2-naph-

## RESULTS

*Determination of 5-hydroxy-1,2-naphthalic acid in feces.*—One mouse was injected with 0.5 mg. of radioactive dibenzanthracene (305,000 counts/min); feces were collected for 4 days and pooled. To each of the acidic fractions resulting from the extraction procedure (fractions B, D, G, and H, Chart 1) was added a known weight of 5-hydroxy-1,2-naphthalic acid—usually about 10 mg. This was then recovered by sublimation and purified by recrystallization and additional sublimations. Recrystallization was carried out on samples of

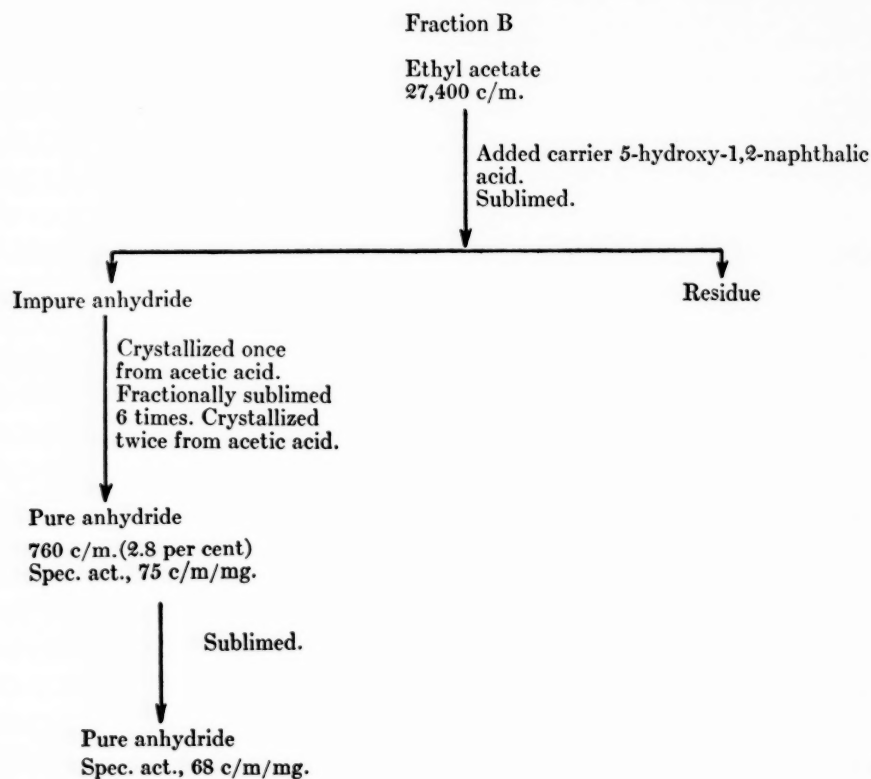


CHART 3.—Purification of fraction B

thalic acid. The conversion of the acid to the anhydride can be accomplished by sublimation or crystallization from benzene.

Absorption spectra analyses of aqueous solutions of Dauben's compound and the compound synthesized here were identical, showing maxima at 295  $m\mu$  and 340  $m\mu$ ; two-dimensional filter paper chromatography with two sets of solvents (phenol, water; and butanol, propionic acid, water) (1) failed to demonstrate any differences when run on the individual compounds and on mixtures of the two. The anhydride, when added as carrier to radioactive acid fractions, failed to maintain radioactivity, but the acid did, showing that the actual metabolite is the acid.

from 5 to 10 mg. with about 2 ml. of solvent. Benzene proved to be the best solvent to remove radioactive impurities and was used in preference to water and acetic acid. Sublimations were performed *in vacuo* (0.01–0.1 mm. and 160°–180°); the anhydride was condensed on a water-cooled cold finger. Chart 3 shows the methods used to purify the carrier added to fraction B. The 5-hydroxy-1,2-naphthalic acid recovered from each of the four acid fractions maintained radioactivity throughout similar purifications, indicating that this compound occurred in both free and conjugated forms in the feces. These results are summarized in Table 1. About 0.4 per cent of the administered hydrocarbon was converted to the metabolite.

To give further evidence of purity, a known weight of carrier, purified to constant specific activity, was equilibrated between two immiscible solvents, ether and dilute acetic acid, contained in a centrifuge tube. The upper phase was drawn off with a capillary pipette, and the volume of each phase was measured. The amount of the anhydride contained in each solvent and its specific

TABLE 1

## RESULTS OF CARRIER EXPERIMENTS ON ACID FRACTIONS FROM FECES

Fraction	Description of fraction	Total counts in fraction	Total counts in carrier	B×100*
		A counts/min	B counts/min	A (per cent)
B	Water-insoluble unconjugated acids	27,400	680	2.8
D	Acids made water-soluble by conjugation	1,800	58	3.2
G	Conjugated acids hydrolyzed by boiling water	6,300	256	4.1
H	Conjugated acids hydrolyzed by boiling NaOH	1,300	92	7.1

\* The acid fraction represents about 95 per cent of the total metabolites in the feces.

Solubility distribution coefficient,  $K$

$$K = \frac{C_1}{C_2} = \frac{\text{mg anhydride/ml of ether}}{\text{mg anhydride/ml of aqueous phase}}$$

$$K = \frac{3.2 \text{ mg}/0.9 \text{ ml}}{1.2 \text{ mg}/1.4 \text{ ml}} = 4.2$$

Radioactivity distribution coefficient,  $H$

$$H = \frac{C_1}{C_2} = \frac{\text{counts/min/ml of ether}}{\text{counts/min/ml of aqueous phase}}$$

$$H = \frac{112 \text{ counts/min}/0.9 \text{ ml}}{39 \text{ counts/min}/1.4 \text{ ml}} = 4.5$$

CHART 4.—Results of partition experiment

activity were determined. From these data, it was possible to determine the solubility distribution coefficient,  $K$ , and the radioactivity distribution coefficient,  $H$ . If the radioactive substance present in minute amounts is identical with the carrier, the values of  $H$  and  $K$  will be equal. Results of this partition experiment are given in Chart 4; the difference is within the experimental error.

These findings were confirmed by experiments involving four mice, each injected with 0.5 mg. of hydrocarbon (305,000 counts/min).

*Determination of 5-hydroxy-1, 2-naphthalic acid in liver.*—Each of three mice was injected with 0.5

mg. of the radioactive hydrocarbon (305,000 counts/min.). One mouse was killed 2 hours after injection; the second and third mice were killed after 6 and 22 hours, respectively. Their livers were removed immediately and treated as previously described. Carrier (1–2 mg.) was added to the acid fractions obtained from the extraction procedure illustrated in Chart 2 and was recovered by sublimation and placed on a filter-paper chromatogram. The spot was developed in two dimensions using two sets of solvents (phenol, water; and butanol, propionic acid, water) (1). The spot was cut from the paper; the carrier was eluted with dilute alkali and extracted into ethyl acetate. Additional carrier was added and was recrystallized from benzene to constant specific activity. The results contained in Table 2 show that the metabo-

TABLE 2

## DETERMINATION OF 5-HYDROXY-1,2-NAPHTHALIC ACID IN LIVER

Duration of experiment (hours)	Total counts injected (counts/min)	Total counts in acid fraction (Sol. 1, Chart 2) (counts/min)	Total counts in purified carrier (counts/min)
2	305,000	18,400	0
6	"	2,900	0
22	"	260	132

lite did not occur in the livers 2 and 6 hours after administration of the carcinogen but could be found after 22 hours.

*Determination of 5-hydroxy-1,2-naphthalic acid in epidermis.*—The hair was shaved from the backs of five mice, and each mouse was treated once with 0.4 mg. of radioactive dibenzanthracene dissolved in benzene (244,000 counts/min) delivered from a capillary pipette. When dry, the treated area was covered with a Band-Aid, and each mouse was placed in a separate cage which restricted its movements. This restriction was necessary to prevent removal of the Band-Aid and oral ingestion of the hydrocarbon. After 11 days the mice were killed, and the skin was removed from the back of each mouse. The epidermis and dermis were separated by a method developed in these laboratories.<sup>3</sup> The five samples of each layer were pooled, and each was pulverized under liquid air in a mortar. The epidermis and dermis were homogenized separately in a Waring Blendor with 15 ml. of water; the water was then removed in a vacuum oven at 30° over sulfuric acid. The samples were taken up in 15 ml. of 85 per cent ethanol containing 10 per cent trichloroacetic acid and again homogenized in a Waring Blendor. The homogenates were centrifuged, and

<sup>3</sup> To be reported in a subsequent article.

the supernatant liquid was poured off. The tissues were suspended in ethanol and again centrifuged. This washing was repeated several times, and the washings were added to the ethanol-TCA solutions. The tissues, wrapped in filter paper, were extracted with hot ethanol in a Soxhlet extractor for 6 hours; the extraction solvents were combined with the ethanol-TCA solutions (14). These combined solutions were submitted to the fractionation scheme outlined in Chart 5.

The activity of the acid solution obtained from dermis was too low to warrant further treatment. The extract of the epidermis was combined with carrier, and the solvent was evaporated. The tri-

recovered 1,2-naphthalic anhydride was accomplished by sublimation; 39.0 mg. were obtained. A small amount of the impure compound was treated with diazotized sulfanilic acid in alkaline solution; no evidence was obtained that hydroxylation of the anhydride had occurred. Under the conditions of this test, 5-hydroxy-1,2-naphthalic acid coupled with the diazonium salt to give an intense red color. The impure anhydride was crystallized once from a mixture of ligroin in benzene (1:3), yielding pure 1,2-naphthalic anhydride, m.p. 164°–166°.

*Tests for carcinogenicity and tumor regression.*—Experiments testing the carcinogenicity of 5-

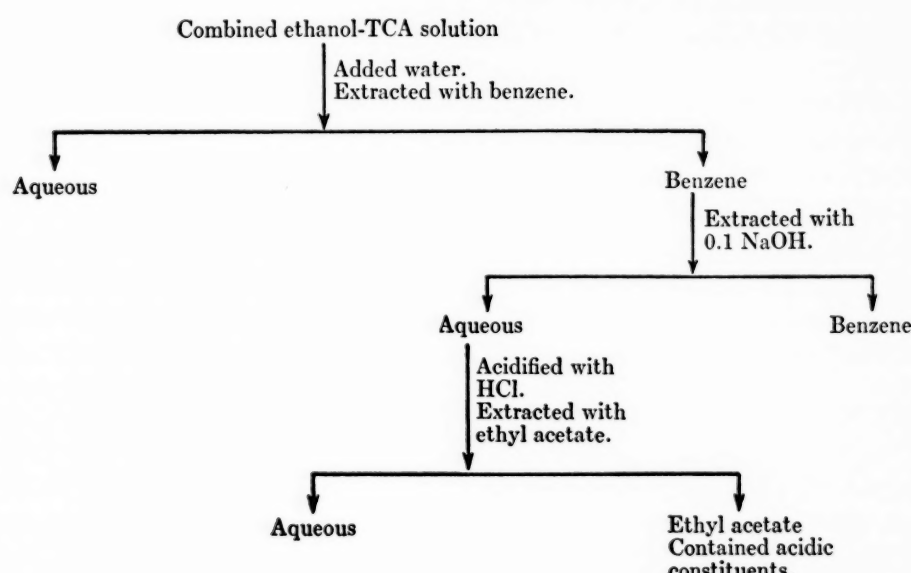


CHART 5.—Fractionation of nonprotein material obtained from skin

chloroacetic acid was removed by fractional sublimation at a low temperature, following which procedure the anhydride was sublimed. It was purified by recrystallization from benzene and chromatography on a column of Florisil. The anhydride was placed on the column in acetone and was eluted with a solution of 10 per cent acetone in ligroin; it chromatographed in a single yellow band and retained constant specific activity throughout two chromatograms.

*Injection of 1,2-naphthalic anhydride.*<sup>4</sup>—Each of four mice was injected intravenously with a solution of 10 mg. of 1,2-naphthalic anhydride neutralized with sodium carbonate. Urine was collected from each mouse for 48 hours, and at the end of that time the samples were pooled. The acidic constituents were removed by suitable extraction procedures. Partial purification of the

<sup>4</sup> Kindly furnished by Prof. L. F. Fieser, Harvard University.

hydroxy-1,2-naphthalic acid and 5-hydroxy-1,2-naphthalic anhydride were carried out for 7 months with negative results.

It has been reported by Haddow (7,8) that chemical carcinogens act as growth inhibitors, not only for normal tissues but for tumor tissue as well. Since the mechanism of this inhibition has not been determined, it seemed possible that the action might be due to a metabolite instead of to the carcinogen itself. For this reason, tumor-bearing rats and mice were injected with neutralized solutions of 5-hydroxy-1,2-naphthalic acid. The compound had no effect on the normal growth rate of the tumors.

## DISCUSSION

The appearance of radioactive 5-hydroxy-1,2-naphthalic acid in the feces of mice following an intravenous injection of 1,2,5,6-dibenzanthracene-9,10-C<sup>14</sup> proves that the acid is metabolically de-

rived from the hydrocarbon. This metabolite is excreted free and in conjugated form. It is combined with substances which render it more water-soluble and with other compounds, possibly proteins, which decrease its solubility. That the formation of the metabolite is not due entirely to the action of intestinal flora is shown by its presence in liver; it is probable that at least part of the total amount of metabolite appearing in the feces is formed in the liver. Since it has been demonstrated (10) that the elimination of radioactivity from the liver into the intestines occurred via the bile, it is probable that the acid is also eliminated by this route.

The demonstration that 1,2-naphthalic anhydride was not converted by the mouse to 5-hydroxy-1,2-naphthalic acid and the fact that the former compound did not carry radioactivity from acid fractions prove that in order for the central ring of the hydrocarbon to be metabolically cleaved, hydroxyl groups must be present in the terminal rings. Dauben and Tanabe (5) suggested that the unsymmetrical cleavage of dihydroxy-dibenzanthracene might proceed through 4',8'-dihydroxydibenzanthracene-9,10-quinone. Plans to test this hypothesis are being considered.

Several other examples of the biological cleavage of aromatic rings have been shown to occur. In 1949, Schepartz and Gurin (16) presented evidence which indicated that homogentisic (or quinone acetic acid) is unsymmetrically cleaved to yield acetoacetate. More recently, Ravdin and Crandall (15), using partially purified enzyme preparations from rat liver, have confirmed this work and have isolated an intermediate in the reaction which they identify as fumarylacetoacetate.

It is of interest that the presence of the metabolite could be demonstrated in mouse epidermis after the administration of dibenzanthracene to the skin; this indicates a high degree of metabolic activity in the epidermis. Even though tumors occur in the epidermal layer following applications of dibenzanthracene to the skin, 5-hydroxy-1,2-naphthalic acid itself has no carcinogenic activity and, therefore, is probably not directly involved in the carcinogenic process. It could be indirectly associated with carcinogenesis, if one assumes a theory, such as Boyland's (2), that carcinogenesis is caused by the energy from the metabolism of the carcinogenic agent. It seems more likely, however, that the occurrence of the metabolite results from the detoxication of dibenzanthracene.

#### SUMMARY

1. The metabolism of 1,2,5,6-dibenzanthracene in the mouse was investigated; this was done with

$C^{14}$ -labeled hydrocarbon and with the aid of certain technics applicable to radioactive isotopes.

2. It has been shown that 5-hydroxy-1,2-naphthalic acid is a metabolite of dibenzanthracene. The acid was found in the feces and liver of mice injected intravenously with the hydrocarbon and in epidermis of mice which had previously been treated on the skin with dibenzanthracene.

3. 5-Hydroxy-1,2-naphthalic acid is not carcinogenic to mice and hence cannot be directly involved in the carcinogenic process.

4. 5-Hydroxy-1,2-naphthalic acid has no tumor-growth inhibitory properties in mice and rats.

5. 1,2-Naphthalic anhydride has been shown not to be a precursor of 5-hydroxy-1,2-naphthalic acid.

6. Evidence has been presented which demonstrates the distinction between 5-hydroxy-1,2-naphthalic acid and 5-hydroxy-1,2-naphthalic anhydride.

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# Folic Acid Analogs and the Growth of Embryo and Tumor Tissue

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Analogs of pteroylglutamic acid (folic acid) have been extensively investigated from both the standpoint of their preparation and their biological effects. Numerous compounds of this type have been synthesized and their effects tested with respect to the growth of various animals and tissues. The present status of this field of research has been summarized in some of the contributions to the recent series of papers on anti-metabolites given under the auspices of the New York Academy of Sciences (2-6, 9).

It is generally considered that these compounds, when introduced into an animal, serve as antagonists of folic acid and especially affect those tissues which are sensitive to an insufficiency of this vitamin. It has also been demonstrated that there is a wide variation in the dosage of the available analogs required to block out folic acid from the cells (4, 5, 7).

A number of folic acid analogs have been tested on malignant neoplasms (9, 10). It has been found that growth inhibition may result from the application of these compounds. 4-Aminopteroylglutamic acid (aminopterin) has proved especially effective in this regard. However, nontumor tissue is also adversely affected. Other less toxic folic acid analogs such as pteroyltriglutamic acid (teropterin) and pteroylaspartic acid (An-Fol-A) have been found to be less effective, even at comparatively large dosages. Stock (9) and associates found that teropterin was ineffective on egg-cultivated tumors when dosages as high as 20 mg. per injection were used. Such data as are available indicate that different types of tumors do not react similarly to such compounds. Sugiura (10) and co-workers have reported that aminopterin had an inhibitory effect at toxic levels on transplants of a sarcoma in rats and on a sarcoma, mammary carcinoma, and melanoma in mice. Spontaneous breast tumors in mice were not affected.

The present investigation was undertaken to test representatives of folic acid analogs on the growth of embryo and yolk sac tumors of tumor-

bearing eggs. Tumors cultivated in this manner grow in the embryonated egg, sharing a common blood supply with the embryo but without otherwise interfering with the embryonic course of development.

This method of growing tumor tissue has been used in this laboratory for more than 8 years (13-15). During that time hundreds of thousands of eggs have been inoculated with tumor tissue in association with various research projects. The techniques involved in the process have been progressively improved and simplified. During the past 2 years yolk sac-cultivated tumors have been used in cancer chemotherapy studies (11, 12). Since the publication of the first report on this work, about 18,000 tumor-bearing eggs have been utilized in testing compounds. It has been shown that tumors grown by the yolk sac method give 100 per cent "takes" and grow as uniformly as implants of the tumor tissue in the natural host. Furthermore, the growth of the tumor used in these experiments is so rapid that 24-hour tests are possible.

Three folic acid analogs,<sup>1</sup> aminopterin, teropterin, and An-Fol-A have been used in a series of tests using embryonated eggs implanted in the yolk sac with a C3H mouse mammary carcinoma. Tests were also made with folic acid, synthetic folinic acid<sup>2</sup> (citrovorum factor), and these compounds in combination with aminopterin and teropterin.

The object of the investigation was to determine the comparable reactivity of tumor and embryo to the test compounds. Data obtained by the same approach with cortisone acetate are included for comparison.

## MATERIALS AND METHODS

Embryonated eggs with yolk sac implants of a C3H mammary tumor were used in these studies. The eggs were inoculated with tumor tissue on about the fourth day of incubation, as previously

<sup>1</sup> Supplied by Lederle Laboratories.

<sup>2</sup> Supplied by Dr. William Shive of the University of Texas Biochemical Institute.

described (13). The tumor grows rapidly and relatively uniformly by this method of cultivation in every inoculated egg.

On the twelfth day of egg incubation (8 days after tumor inoculation), the compound to be tested was introduced between the shell membranes and the blood vessels of the chorioallantoic membrane and area vasculosa. This was done by hypodermic injection through a dented area in the shell, the details of which have been described (11).

The compounds used in these experiments were injected, in most instances, as aqueous solutions or suspensions in combination with cottonseed oil. One part of the aqueous solution or suspension was thoroughly mixed with 2 parts of the oil, and 0.2 cc. of the emulsion was introduced into the experimental eggs. This was done to prevent too rapid absorption and excretion of the test compound into the allantoic fluid. The control eggs were injected with the suspending fluid.

The tumor weights averaged 0.21–0.40 gm. per egg on the twelfth day of incubation. In the next 24 hours these yolk sac tumors increased an average of 70 per cent in size. The embryos supporting the tumors averaged 2.9–3.4 gm. and gained in weight an average of 30 per cent for the same time interval. This 24-hour period was used for testing the effects on the growth of tumor and embryo of the injected compound.

Each experiment was carried out as follows:

Twelve-day tumor-bearing eggs were divided into comparable groups of about 15 eggs each. One group was harvested at once to obtain the average tumor and embryo weight at the beginning of the experiment. The other eggs were divided into experimental and control groups and injected with the appropriate material. The eggs were incubated at 37° C. for 24 hours. The embryos and tumors were then harvested and weighed individually. Representative samples of control and experimental tumors were selected for histological examination.

The effect on growth was evaluated on the basis of the increase in size of embryo and tumor which occurred in the control eggs during the period of the experiment.

Teropterin was used in sixteen experiments involving a total of 248 tumor-bearing eggs, aminopterin in ten experiments for a total of 240 eggs, and An-Fol-A was the test compound in nine experiments for a total of 270 egg injections. A series of twelve experiments utilizing 206 tumor-bearing eggs was made with folic acid, folinic acid, and these compounds in combination with aminopterin and teropterin. Cortisone acetate was tested in six experiments for a total of 192 tumor-bearing eggs.

Comparable nontumor eggs were used in a number of experiments to check the effect of the compounds on the embryo uncomplicated by the presence of a tumorous growth in the yolk sac.

## RESULTS

The principal data are summarized in Tables 1 and 2. The statistical validity of these results is evident.

It will be noted in Table 1 that teropterin was a more effective inhibitor of tumor growth under the conditions of the experiments than aminopterin. Furthermore, the effect on the tumor was obtained with very little disturbance to the embryo. It is also evident that dosage was an important factor in the experiment with teropterin. Tumor growth showed a maximum inhibition when 0.1 mg. of the compound per injection was used. Lower dosages not recorded in the table were used in a few experiments but were less effective. A number of experiments were carried out with teropterin at various levels in solution without the admixture of cottonseed oil. Under those circumstances the effect on tumor and embryo growth was negative. Apparently, the injected material was taken up by the blood and passed into the allantoic fluid too rapidly to be effective. Tests were also made with nontumor embryonated eggs. These embryos were not affected by dosages used for the tumor-bearing egg injection.

The effect of teropterin on tumor growth was almost entirely eliminated when administered in association with either folic acid or folinic acid (Table 2). The data indicate, however, that there was still more inhibition of tumor growth than was true for either folic acid or folinic acid alone.

An-Fol-A resembled teropterin at the 1.0-mg. per injection level. Lower levels were ineffective. This compound was also relatively nontoxic to the chick embryo.

Aminopterin at subtoxic dosages had little effect on tumor or embryo growth. At a dosage which gave evidence of disturbance to the embryo, tumor growth was significantly inhibited.

Folic acid in combination with aminopterin reduced the inhibitory effect on tumor growth with possibly some protective action on the embryo. The data indicate that folinic acid, when administered with aminopterin, accentuated the inhibitory effect on tumor growth with about the same effect on the embryo as the folic acid-aminopterin combination.

Cortisone acetate, as the table shows, was used at levels varying from 0.1 to 1.5 mg. per injection. At all these dosages the growth of tumor and embryo was inhibited and to about the same degree.

Histological examination of the tumor tissue showed no necrosis or other evidence of toxicity. The tumor cells appeared to be unaffected in association with almost complete inhibition of growth. As further evidence of this, when eggs treated with the folic acid analogs were allowed to continue their development with no further injections, the yolk sac tumors resumed their usual rapid growth.

### DISCUSSION

The use of yolk sac cultivated tumors makes it possible to compare the reactions of neoplastic and rapidly proliferating non-neoplastic tissue to a particular compound. The relationship of the yolk sac tumors and the host embryo are such that, in the early stages, they maintain vigorous and independent growth and at the same time

share a common blood stream and a common environment.

The method of introducing the test compound into the egg which was used in these experiments ensures absorption into the chick blood system. In a recent paper Galinsky (1) demonstrated that radioactive phosphorus injected by this technic over the chorioallantoic membranes and area vasculosa was taken up by the blood stream and distributed to the chick embryo, yolk sac tumor, and other parts of the egg.

The rate of absorption naturally varies with the type of material injected. In the present investigation the best results were obtained when absorption of the inoculum was made to proceed more slowly by the use of cottonseed oil in the suspending medium.

TABLE 1

THE EFFECT OF FOLIC ACID ANALOGS AND CORTISONE ACETATE ON THE GROWTH OF TUMOR AND EMBRYO OF TUMOR-BEARING EGGS  
(24-Hour Test Period)

		SURVIVAL		AV. INITIAL WEIGHT		AV. TUMOR WEIGHT, END OF TEST PERIOD		AV. EMBRYO WEIGHT, END OF TEST PERIOD		GROWTH INHIBITION	
No. EGGS	No. EXPS.	CONTROL =100	DOSAGE (MG.)	Tumor (gm.)	Embryo (gm.)	Exper. (gm.)	Control	Exper. (gm.)	Control	Tumor (per cent)	Embryo
Teropterin											
92	3	97	1.0	0.34±0.06	3.07±0.21	0.46±0.07	0.58±0.07	3.93±0.19	3.99±0.27	50	7
96	3	100	0.5	0.39±0.02	3.39±0.15	0.47±0.03	0.66±0.02	4.36±0.09	4.40±0.17	73	5
136	4	100	0.1	0.39±0.04	3.15±0.12	0.44±0.03	0.68±0.06	4.03±0.16	4.09±0.14	83	6
Aminopterin											
92	3	100	0.15	0.35±0.07	3.13±0.22	0.43±0.08	0.59±0.12	3.90±0.26	4.06±0.28	67	15
92	3	99	0.1	0.35±0.05	2.90±0.16	0.56±0.09	0.59±0.06	3.71±0.16	3.80±0.14	12	11
An-Fol-A											
156	5	101	1.0	0.35±0.07	3.00±0.20	0.46±0.04	0.60±0.11	3.80±0.15	3.86±0.23	56	7
Cortisone acetate											
160	5	100	0.1-1.5	0.43±0.03	3.26±0.15	0.59±0.04	0.72±0.05	3.80±0.12	4.33±0.07	43	50

TABLE 2

THE EFFECT OF FOLIC ACID AND FOLINIC ACID, ALONE AND IN COMBINATION WITH TEROPTERIN AND AMINOPTERIN ON THE GROWTH OF TUMOR AND EMBRYO OF TUMOR-BEARING EGGS  
(24-Hour Test Period)

MATERIAL INJECTED	DOSAGE (MG.)	No. EXPS.	No. EGGS	AV. INITIAL WEIGHT		AV. TUMOR WEIGHT, END OF TEST PERIOD		AV. EMBRYO WEIGHT, END OF TEST PERIOD		GROWTH INHIBITION	
				Tumor (gm.)	Embryo (gm.)	Exper. (gm.)	Control (gm.)	Exper. (gm.)	Control (gm.)	Tumor (per cent)	Embryo (per cent)
Folic acid	1.0-3.0	3	66	0.38	4.0	0.56±0.08	0.64±0.09	5.33±0.33	5.20±0.04	31	-10*
Folic acid	0.10	1	16	0.21	3.1	0.46±0.06	0.49±0.05	4.28±0.10	4.48±0.20	11	13
Folinic acid	0.10	1	16	0.21	3.1	0.47±0.07	0.49±0.05	4.27±0.12	4.48±0.20	7	13
Folic acid	0.10	1	16	0.21	3.1	0.43±0.06	0.49±0.05	4.42±0.08	4.48±0.20	21	4
Teropterin	0.10										
Folic acid	0.10	1	16	0.24	3.4	0.38±0.06	0.51±0.08	4.66±0.15	4.52±0.12	49	-12*
Aminopterin	0.20										
Folic acid	0.10	1	16	0.21	3.3	0.31±0.03	0.41±0.12	4.26±0.22	4.41±0.18	49	15
Aminopterin	0.15										
Folinic acid	0.10	1	16	0.21	3.1	0.31±0.08	0.49±0.05	4.32±0.16	4.48±0.20	64	11
Aminopterin	0.10										
Folinic acid	0.10	1	16	0.21	3.3	0.24±0.08	0.41±0.12	4.45±0.20	4.41±0.18	85	-3*
Aminopterin	0.15										
Folinic acid	0.10	1	16	0.24	3.4	0.28±0.09	0.51±0.08	4.38±0.27	4.52±0.12	85	12
Aminopterin	0.20										
Folinic acid	0.10	1	16	0.24	3.4	0.44±0.12	0.51±0.08	4.51±0.24	4.52±0.12	27	3
Teropterin	0.10										

\* Indicates per cent growth acceleration.

The data indicate that an egg-cultivated mammary carcinoma was more sensitive to certain folic acid analogs than the associated chick embryo. This was particularly true with respect to teropterin and An-Fol-A. Such a difference in the reaction of embryo and tumor of tumor-bearing eggs to an introduced compound is very uncommon. Several hundred compounds have been tested to date, and in most instances there is either no effect, or the tumor and embryo react similarly. The usual outcome of such experiments is exemplified in the data on cortisone acetate. This compound inhibited both types of tissue to about the same degree.

The results obtained when folic acid and folinic acid were used in combination with either aminopterin or teropterin are of some interest since they indicate, as did the reaction of the tumor to teropterin, a possible difference in the biochemistry of the tumor, as compared to the embryo. This is particularly true with respect to the effect on tumor growth obtained by a combination of aminopterin and folinic acid.

Snell and Cravens (8) reported that concentrates of the citrovorum factor did not lessen the inhibitory effect of aminopterin on chick embryos. They also obtain negative results in this respect with folic acid. In the present investigation folinic acid not only failed to reverse the effect of aminopterin but increased the inhibitory effect on tumor growth to the point where it equaled that of teropterin.

A more extended investigation of the role of folinic acid on tumor and embryo biochemistry is contemplated.

#### SUMMARY

Folic acid analogs were introduced over the chorioallantoic membrane and area vasculosa of 12-day embryonated eggs having yolk sac implanted mouse mammary carcinoma. A total of 35 experiments involving 758 tumor-bearing eggs were utilized.

Teropterin inhibited tumor growth an average of 50, 73, and 83 per cent, at dosages of 1.0, 0.5, and 0.1 mg. per injection. Growth of the associated chick embryos was only slightly affected.

Aminopterin inhibited tumor growth an average of 67 and 12 per cent at levels of 0.15 and 0.1 mg. per injection. The growth of the chick embryos was inhibited 11 and 15 per cent, with evidence of some toxicity at the higher level.

An-Fol-A at 1.0 mg. per injection inhibited tumor growth an average of 56 per cent, with the chick embryos very slightly affected. Lower dosages were ineffective.

The effect of folic acid and folinic acid alone and in combination with teropterin and aminopterin was tested. Each of these compounds in 0.1-mg. doses slightly inhibited tumor and embryo growth. Both folic acid and folinic acid counteracted the tumor growth inhibitory effect of teropterin. Folic acid had little or no effect on the tumor growth inhibitory action of aminopterin. Folinic acid in combination with aminopterin was more inhibitory of tumor growth than aminopterin alone.

Microscopic examinations of tumor tissues disclosed no evidence of histological or cytological disturbance.

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# The Intracellular Distribution of Protein, Nucleic Acids, and Riboflavin in the Livers of Mice and Hamsters Fed 4-Dimethylaminoazobenzene\*

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Previous studies have shown that the ingestion of the hepatic carcinogen 4-dimethylaminoazobenzene by rats causes alterations in the levels and intracellular distribution of several constituents of the liver (9-11). Thus, there was an increase in the desoxypentosenucleic acid and protein contents of the nuclear fraction, a marked decrease in the amounts of protein, riboflavin, and pentosenucleic acid in the large granule (mitochondria) fraction, and a large decrease in the pentosenucleic acid content of the small granule (microsome) fraction. These changes in the morphological fractions were found long before neoplasms were detectable by gross or microscopic examination of the organ and tended to make the composition of the liver resemble that of the malignant hepatic tumors eventually produced by the dye (11-13).

The number of tumors induced by 4-dimethylaminoazobenzene can be altered markedly by changes in diet, the structure of the dye, or the species used (2, 5, 6, 15), and these variations have been used to outline the relationship of the intracellular changes to the carcinogenic process. Thus, when this dye was fed to rats in a diet containing a level of riboflavin high enough to inhibit strongly the development of liver tumors, no changes from normal were found in the nuclear fraction (9). However, the alterations in the levels of protein and pentosenucleic acid in the large and small granules were the same whether a high or low level of riboflavin was fed with the dye. Similarly, analyses were made on the livers of rats fed a series of seven aminoazo dyes closely related in structure to 4-dimethylaminoazobenzene but with carcinogenic activities ranging from 0 to 12 (activity of 4-dimethylaminoazobenzene = 6 [11, 12]). In general, the effects of these aminoazo dyes on the composition of rat liver were qualitatively the same as

those of the parent compound, and the degree of change was approximately proportional to the carcinogenic potency of the derivative fed.

In the present paper these comparisons are extended to the livers of two species, the mouse and the golden hamster, which are highly resistant to the carcinogenic action of 4-dimethylaminoazobenzene. With this agent mice develop tumors only slowly (5, 15) and hamsters not at all (7); hence, one might expect that this aminoazo dye would have a slight to moderate effect on the composition of mouse liver and little or no effect on the composition of hamster liver. Since the reported intracellular distribution of desoxypentosenucleic acid in the livers of C3H mice (18) differs from that obtained in these experiments with an unknown strain of albino mice, comparative data on the fractionation of livers from these two strains are also presented.

## METHODS

Adult female albino mice<sup>1</sup> and adult male hamsters (Golden Syrian)<sup>2</sup> were fed a semi-synthetic diet (6, diet 3) containing 12 per cent of casein and 1.2 mg riboflavin/kg, with or without the addition of 4-dimethylaminoazobenzene, for 4 months. The diet for the hamsters contained 0.06 per cent of dye, the level fed to rats in previous experiments (9, 11), while the diet for the mice contained only 0.045 per cent, since the mortality is high when greater amounts are fed (2). The adult C3H mice<sup>3</sup> were of mixed sex and were maintained on a stock diet<sup>4</sup> without the addition of dye.

Duplicate or triplicate fractionations were made in each case, and six to nine mice or three hamsters were used for each fractionation. The animals were killed with ether, and their livers were immediately perfused *in situ* with 0.14 M NaCl. The per-

<sup>1</sup> Obtained from A. H. Sutter, Springfield, Mo.

<sup>2</sup> Obtained from J. C. Landis, Hagerstown, Md.

<sup>3</sup> Obtained from the Bar Harbor Laboratories, Bar Harbor, Me.

<sup>4</sup> Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo.

\* This work was supported in part by a grant from the National Cancer Institute, Public Health Service.

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fusion and all subsequent steps prior to analysis were carried out at 0°–5° C. The livers were pooled, forced through a plastic tissue mincer, and homogenized in 0.88 M sucrose solution (9). Nuclear, large granule, small granule, and supernatant fluid fractions were prepared by differential centrifugation (9, 11).

The nucleic acids were determined according to the method of Schneider (16), and protein was determined gravimetrically (9). Riboflavin was assayed by the acid production of *Lactobacillus casei* (14). Protein-bound aminoazo dye was determined

the carcinogen (9, 11), while no change in the level of protein in the supernatant fluid was observed when 4-dimethylaminoazobenzene was fed to rats. Ingestion of the dye had no significant effect on the levels or intracellular distribution of protein in the hamster livers (Table 1).

*Distribution of desoxypentosenucleic acid.*—There appeared to be little or no change in the desoxypentosenucleic acid content of either mouse or hamster liver following the ingestion of 4-dimethylaminoazobenzene (Table 2). However, the values obtained for the livers from the dye-fed

TABLE 1  
DISTRIBUTION OF PROTEIN IN THE LIVER FRACTIONS

LIVER FRACTION	ALBINO MOUSE LIVERS*		HAMSTER LIVERS†			
	Basal	Basal+dye	Basal	Basal+dye	Basal	Basal+dye
	Mg. of protein per gram of fresh liver (The figures to the nearest whole numbers)					
Whole homogenate	131 (123–141)	133 (125–142)	130	121	134	133
Nuclei	23 (16–30)	30 (28–35)	28	18	24	24
Large granules	35 (34–35)	23 (22–25)	30	31	33	30
Small granules	16 (15–18)	15 (12–16)	17	15	17	19
Supernatant fluid	53 (50–57)	63 (61–67)	56	55	59	58
Recovery	127 (119–139)	131 (127–140)	131	119	133	131

\* Average and range of three fractionations.

† Each figure refers to one fractionation.

TABLE 2  
DISTRIBUTION OF DESOXPENTOSENUCLEIC ACID IN THE LIVER FRACTIONS

LIVER FRACTION	ALBINO MOUSE LIVERS*		HAMSTER LIVERS*			
	Basal	Basal+dye	Basal	Basal+dye	Basal	Basal+dye
	Mg. of nucleic acid per gram of fresh liver					
Whole homogenate	2.57 (2.42–2.70)	2.48 (2.23–2.77)	1.65	2.00	1.67	1.65
Nuclei†	2.50 (2.19–2.70)	3.03 (2.88–3.27)	1.75	1.93	1.71	1.64
	Mg. of nucleic acid per gram of protein (The figures to the nearest whole numbers)					
Whole homogenate	20 (19–20)	19 (17–20)	13	17	12	12
Nuclei	114 (90–137)	100 (93–105)	63	107	71	68

\* See footnotes to Table 1.

† The other fractions did not contain detectable amounts of this nucleic acid.

as previously described (5). For histological study small blocks of each liver were fixed in Mossman's fluid (3), and sections were stained with hematoxylin and eosin (4). In addition, some sections were stained for desoxypentosenucleic acid by the Feulgen method or for iron by the method of Tirmann and Schmelzer (4).

## RESULTS

*Protein distribution.*—The livers of mice fed 4-dimethylaminoazobenzene contained about 30 and 19 per cent more protein in the nuclear and supernatant fluid fractions, respectively, and about 34 per cent less protein in the large granules than was found in the corresponding fractions of the normal livers (Table 1). The alterations in the protein contents of the nuclear and large granule fractions were similar to those found in the livers of rats fed

mice are somewhat equivocal, since the figures for the nuclear fractions were always about 25 per cent higher than those for the whole homogenate. The cause of these anomalous results is not known, but it may be related to the pigment which was contained in the trichloroacetic acid extracts of the nucleic acids from these livers. No pigment has been encountered in the samples from normal livers.

*Pentosenucleic acid distribution.*—All the cytoplasmic fractions from the livers of mice fed 4-dimethylaminoazobenzene contained less pentosenucleic acid than the same fractions from the livers of control mice (Table 3). These reductions averaged 55 per cent in the case of the large granules, 31 per cent for the small granules, and 19 per cent for the supernatant fluid. The changes in the composition of the granules after dye-feeding were

similar to those found in rats fed the dye. No decrease in the level of pentosenucleic acid in the supernatant fluid was found following ingestion of the carcinogen by rats (9, 11). The recoveries of pentosenucleic acid in the four fractions from the livers of mice fed the dye averaged only 87 per cent, while an average recovery of 95 per cent was obtained with the livers of normal mice. These

the unfractionated tissue. However, while this ratio in each fraction of rat liver was essentially unaltered by ingestion of the dye, the ratio of pentosenucleic acid to protein was reduced in all the cytoplasmic fractions of the livers from mice fed the carcinogen. The ratio of pentosenucleic acid to protein in the nuclear fraction from the hamster livers was somewhat higher than the cor-

TABLE 3

## DISTRIBUTION OF PENTOSENUCLEIC ACID IN THE LIVER FRACTIONS

LIVER FRACTION	ALBINO MOUSE LIVERS*		HAMSTER LIVERS*	
	Basal	Basal+dye	Basal	Basal+dye
	Mg. of nucleic acid per gram of fresh liver			
Whole homogenate	6.54 (5.55-7.37)	5.13 (4.90-5.58)	4.64	4.30
Nuclei	0.74 (0.43-0.91)	0.83 (0.57-1.06)	1.19	†
Large granules	1.63 (1.48-1.81)	0.73 (0.56-0.90)	1.02	1.03
Small granules	1.80 (1.48-2.00)	1.23 (1.03-1.33)	1.44	1.39
Supernatant fluid	2.05 (1.95-2.21)	1.66 (1.43-1.89)	0.99	0.79
Recovery	6.23 (5.39-6.71)	4.45 (4.13-5.00)	4.64	3.70
	Mg. of nucleic acid per gram of protein (The figures to the nearest whole numbers)			
Whole homogenate	50 (45-52)	38 (37-39)	36	35
Nuclei	32 (27-40)	27 (20-31)	43	27
Large granules	47 (42-52)	31 (25-36)	34	33
Small granules	111 (99-122)	84 (83-86)	85	93
Supernatant fluid	39 (38-39)	26 (23-28)	18	14

\* See footnotes to Table 1.

† Sample lost.

TABLE 4

## DISTRIBUTION OF RIBOFLAVIN IN THE LIVER FRACTIONS\*

LIVER FRACTION	ALBINO MOUSE LIVERS		HAMSTER LIVERS	
	Basal	Basal+dye	Basal	Basal+dye
	μg. of riboflavin per gram of fresh liver			
Whole homogenate	18.9	16.1	13.8	9.3
Nuclei	4.0	2.5	3.1	2.0
Large granules	8.9	8.4	4.0	3.3
Small granules	2.4	1.5	1.7	1.3
Supernatant fluid	4.4	2.8	4.1	3.4
Recovery	19.7	15.2	12.9	10.0
	μg. of riboflavin per gram of protein (Figures to the nearest whole numbers)			
Whole homogenate	134	125	97	70
Nuclei	133	109	89	71
Large granules	262	240	182	132
Small granules	133	94	106	109
Supernatant fluid	77	54	61	55

\* Each figure refers to one fractionation.

lower recoveries in the case of the livers from the dye-fed mice may be the result of interference with the orcinol reaction for pentose by the pigment discussed above. The pentosenucleic acid content of hamster liver and its intracellular distribution were not altered by feeding 4-dimethylaminoazobenzene (Table 3).

The ratio of pentosenucleic acid to protein in the fractions from normal mouse liver was similar to that found in normal rat liver (11, 12). In both species the ratio of pentosenucleic acid to protein was lower in the nuclear and supernatant fluid fractions and higher in the small granules than in

responding fraction from the livers of the other two species, while the ratio of these two constituents in the cytoplasmic fractions tended to be lower in the case of hamster liver than for the other species.

**Riboflavin distribution.**—The livers from mice fed 4-dimethylaminoazobenzene contained only 66 per cent as much riboflavin per gram of fresh tissue as normal mouse liver (Table 4). As in the case of rats ingesting the carcinogen (9, 11), the greatest decrease in riboflavin content occurred in the large granule fraction. With the hamsters, on the other hand, there was no reduction in the amount of

hepatic riboflavin following ingestion of the dye. In fact, the large granules from the livers of hamsters fed the dye appeared to contain more riboflavin than the same fraction from normal livers.

With both of these species and the rat (11, 12), the ratio of riboflavin to protein was greater in the large granule fraction and much lower in the supernatant fluid than in the unfractionated tissue. However, unlike the findings with the rat, the reduction of the riboflavin content of the large granule fraction of mouse liver under the influence of the carcinogen was relatively greater than was the reduction in protein content, so that the ratio of riboflavin to protein was reduced in this fraction.

*Protein-bound dye content of livers.*—In confirmation of earlier results (7), no protein-bound dye was found in the livers or in any liver fraction from hamsters fed the dye. While a low level of bound dye appeared to be present in the livers of mice fed 4-dimethylaminoazobenzene, the nonspecific absorption of the extracts containing the aminoazo dyes released from the protein was so great that the analyses had no quantitative significance. This nonspecific absorption may have been derived from the pigment seen in the livers microscopically, and the increase in the dye-feeding period from 2 months (5) to 4 months may account for the greater interference in this study.

*Histological observations.*—On microscopic examination the livers from the mice fed the basal diet appeared essentially normal. However, in a few sections a slight fatty infiltration was seen throughout the lobules, and occasionally slight round cell and polymorphonuclear leukocytic infiltrations were found in the portal areas. Each of these changes occurred to a greater degree in the livers from mice fed the dye. In addition, there was a slight proliferation of the bile ducts following dye-feeding; these ducts appeared normal except that they were larger than the normal ducts of the portal triad. About one-half of the livers from the mice fed the carcinogen showed no necrotic areas; small areas of necrosis, which involved up to about 5 per cent of the parenchymal mass, were found in the other sections. In some instances the necrotic cells, though morphologically intact, had lost all the basophilic staining qualities of the cytoplasm and nucleus, and took a uniform pink stain with eosin. In other areas the cells had undergone partial to complete dissolution, leaving a reticulum framework which held an inflammatory exudate with cellular debris and polymorphonuclear leukocytes. While the nuclei of normal mouse liver varied considerably more in size than those of either normal rat or hamster livers, the variation in nuclear diameter was even greater in the livers

from mice fed the aminoazo dye. An occasional nucleus in the normal mouse livers contained small acidophilic inclusion bodies, and similar bodies were found in far greater numbers in the nuclei of the livers from mice fed the carcinogen. These bodies were round, generally hyaline in appearance, and varied greatly in size. Some were as small as  $1\mu$  in diameter, but others were noted which nearly filled the nucleus (Fig. 1). Occasionally, three or four were seen in a single nucleus. The bodies were Feulgen-negative, and did not take the Feulgen stain in the control sections where the acid hydrolysis was omitted. They did not contain histochemically detectable iron.

The livers of all the mice fed the carcinogen contained considerable quantities of brownish, intracellular, granular pigment.<sup>5</sup> This pigment could be seen in nearly every lobule (chiefly in the periphery); it was localized largely in the Kupffer cells, although some was found in the cytoplasm of the parenchymal cells as well. An occasional cell was nearly filled with this pigment, which was probably responsible for the dark brown color of the perfused livers from mice fed the dye and for the difficulties encountered in some of the analyses. This pigment contained histochemically detectable iron (Fig. 2). Neither the pigment nor iron-staining granules were found in the livers from mice fed the basal diet. This iron-containing pigment is apparently similar to the one found by Edwards and White (1) in the livers of rats fed 4-dimethylaminoazobenzene, but it was present in far larger quantities in the livers of mice fed this carcinogen.

No alterations from normal were found in the microscopic appearance of the livers from hamsters fed 4-dimethylaminoazobenzene.

*Distribution of nucleic acids and protein in the livers of C3H mice.*—In the above experiments with albino mice essentially all the desoxypentose nucleic acid was found in the nuclear fraction, while Schneider, Hogeboom, and Ross (18) found a significant amount of the desoxypentose nucleic acid from C3H mouse livers in the large and small granules. To determine whether this difference might be related to the strain of mouse employed, two fractionations were made of the livers from C3H mice. As seen in Table 5, essentially all the desoxypentose nucleic acid was again found in the nuclear fraction. Apparently, then, the discrepancy in results must be related to differences in technic.

The distribution of protein and nucleic acids in

<sup>5</sup> Drs. J. W. Wilson and E. Shelton of Brown University have noted a similar pigment in the livers of mice fed 3'-methyl-4-dimethylaminoazobenzene (personal communication).

the livers of C3H mice was similar to that in the livers of the albino mice fed the basal diet (Tables 1-3, 5). However, in these fractionations relatively more pentosenucleic acid was found in the nuclear and large granule fractions and relatively less in the small granule fraction than was reported by Schneider *et al.* (18). We have also differed from these workers in finding more pentosenucleic acid in the large granules of rat liver. While Schneider and Hogeboom (17) have suggested that the difference may be due to a contamination of the large granule fraction with the small granules in the fractionations carried out in this laboratory, other factors must also be considered. These include differences in the experimental diets, in whether or

dye was similar to that observed with rats; however, in the case of mouse liver the supernatant fluid also contained reduced quantities of this component when the carcinogen was fed. In both species there was a striking reduction in the riboflavin content of the large granule fraction following the ingestion of the carcinogen. Similarly, the levels of vitamin B<sub>6</sub> in the large granule and supernatant fluid fractions of rat and mouse livers were decreased following the feeding of 4-dimethylaminoazobenzene (10). Thus, following the ingestion of the carcinogen by the mouse, some changes, especially those in the composition of the large granules, were as great as those found in rat liver. However, the mice were fed the carcinogen for 4 months,

TABLE 5

DISTRIBUTION OF PROTEIN AND NUCLEIC ACIDS IN THE LIVERS OF C3H MICE\*

LIVER FRACTION	PROTEIN		DESOXYPENTOSENUCLEIC ACID		PENTOSENUCLEIC ACID	
			Mg. per gram of fresh tissue			
Whole homogenate	143	148	2.46	2.41	5.85	7.57
Nuclei	31	34	2.27	2.58	1.32	1.75
Large granules	40	40			1.64	2.08
Small granules	13	15			1.74	2.03
Supernatant fluid	57	58			1.49	1.78
Recovery	141	147	2.27	2.58	6.19	7.64

\* Each figure refers to one fractionation.

not the livers are perfused and forced through a tissue mincer to remove most of the nonparenchymal tissue prior to homogenization, and in the concentration of sucrose used.

### DISCUSSION

Some correlation is evident between the susceptibilities of the livers of rats, mice, and hamsters to the carcinogenic action of 4-dimethylaminoazobenzene and the effects of this dye on the intracellular distribution of protein, nucleic acids, and riboflavin in the livers. Thus, the composition of the livers of hamsters, which are resistant to the carcinogenic action of the dye, was essentially unaltered following ingestion of the dye. On the other hand, 4-dimethylaminoazobenzene caused several changes in the composition of mouse liver, and many of these alterations were similar to those produced by the carcinogen in the liver of the rat, a much more susceptible species (9, 11). Thus, the increase in the protein content of the nuclear fraction and the decrease in the protein content of the large granules were almost as great for mice fed the dye as for rats. Unlike rat livers, however, the livers of mice ingesting the dye contained more protein in the supernatant fluid than livers from mice fed the basal diet. The decrease in the pentosenucleic acid content of the large and small granules following administration of the aminoazo

while the changes noted for rats were obtained after only 1 month of dye feeding.

The intracellular distribution of protein, nucleic acids, and riboflavin in the normal livers of the three species is quite similar. The desoxypentose nucleic acid was found exclusively in the nuclear fractions of all three species, and the absolute levels were similar. The hamster livers contained a lower level of pentosenucleic acid in the unfractionated tissue than either rat or mouse liver, but the intracellular distribution was similar.

It might have been preferable to express the data in terms of units per cell or per nucleus (8). However, some of the experiments in this report were carried out before nuclear counts were routinely made, and the rest of the data in this series have been reported in terms of units per gram of fresh tissue.

### SUMMARY

1. Sucrose homogenates of the livers from albino mice or from hamsters fed either a semi-synthetic diet or the same diet plus the hepatic carcinogen 4-dimethylaminoazobenzene were separated by differential centrifugation into nuclear, large granule (mitochondria), small granule (microsome), and supernatant fluid fractions. Homogenates of the livers of C3H mice fed a stock diet were similarly fractionated. The unfractionated homogenates

and the four fractions were analyzed for protein, nucleic acids, and riboflavin.

2. The livers from hamsters, which are refractory to the carcinogenic action of this aminoazo dye, were unaffected following ingestion of the compound for 4 months, except that there was an increase in the riboflavin content of the large granule fraction.

3. Mouse liver, which is slightly susceptible to the carcinogenic action of 4-dimethylaminoazobenzene, was altered considerably by the ingestion of this aminoazo dye for 4 months. There were decreased levels of protein, pentosenucleic acid, and riboflavin in the large granules comparable to those previously found in rat liver. However, the increase in the protein content and the decrease in the pentosenucleic acid content of the supernatant fluid were not found in liver from rats fed the dye.

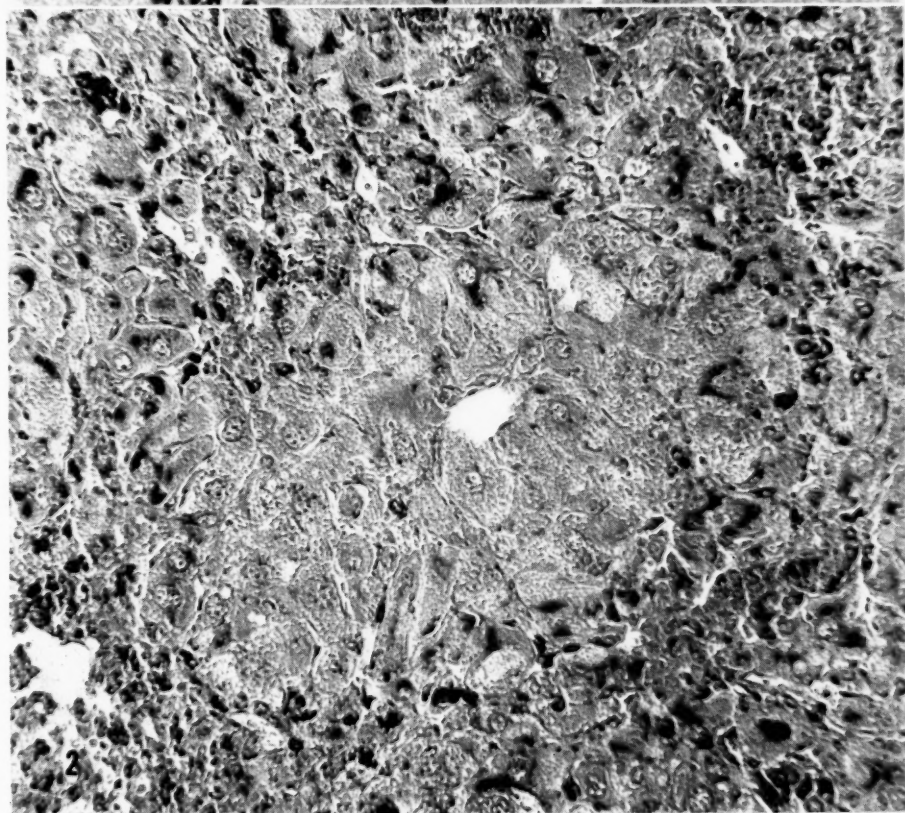
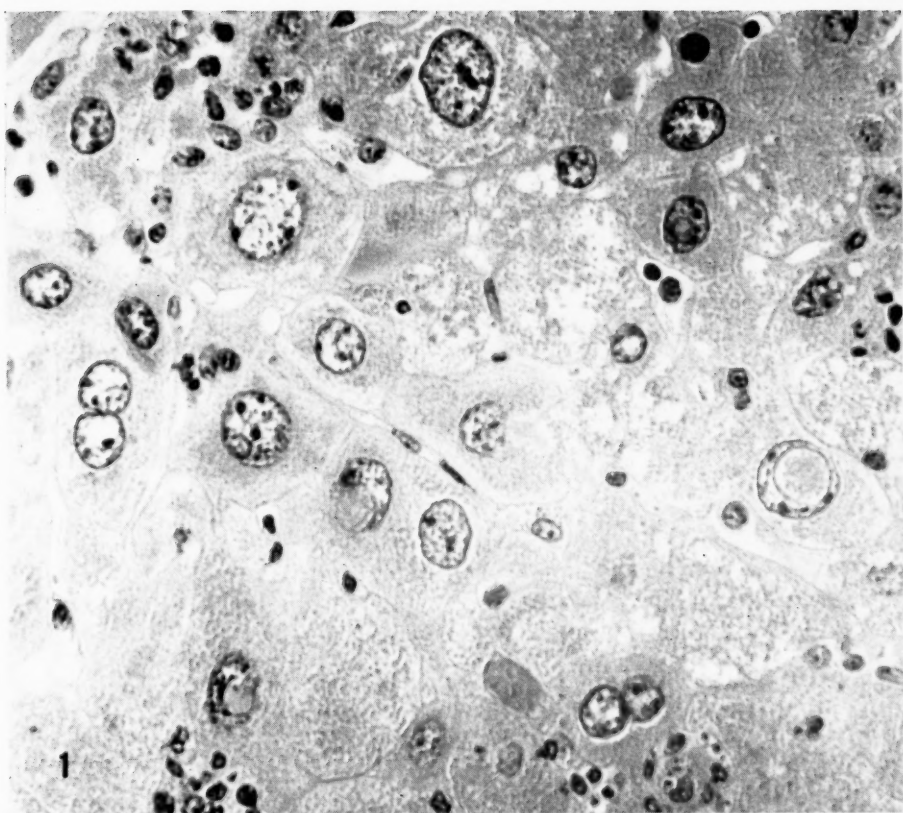
4. The intracellular distribution of protein, nucleic acids, and riboflavin was essentially the same in normal mice, rats, and hamsters.

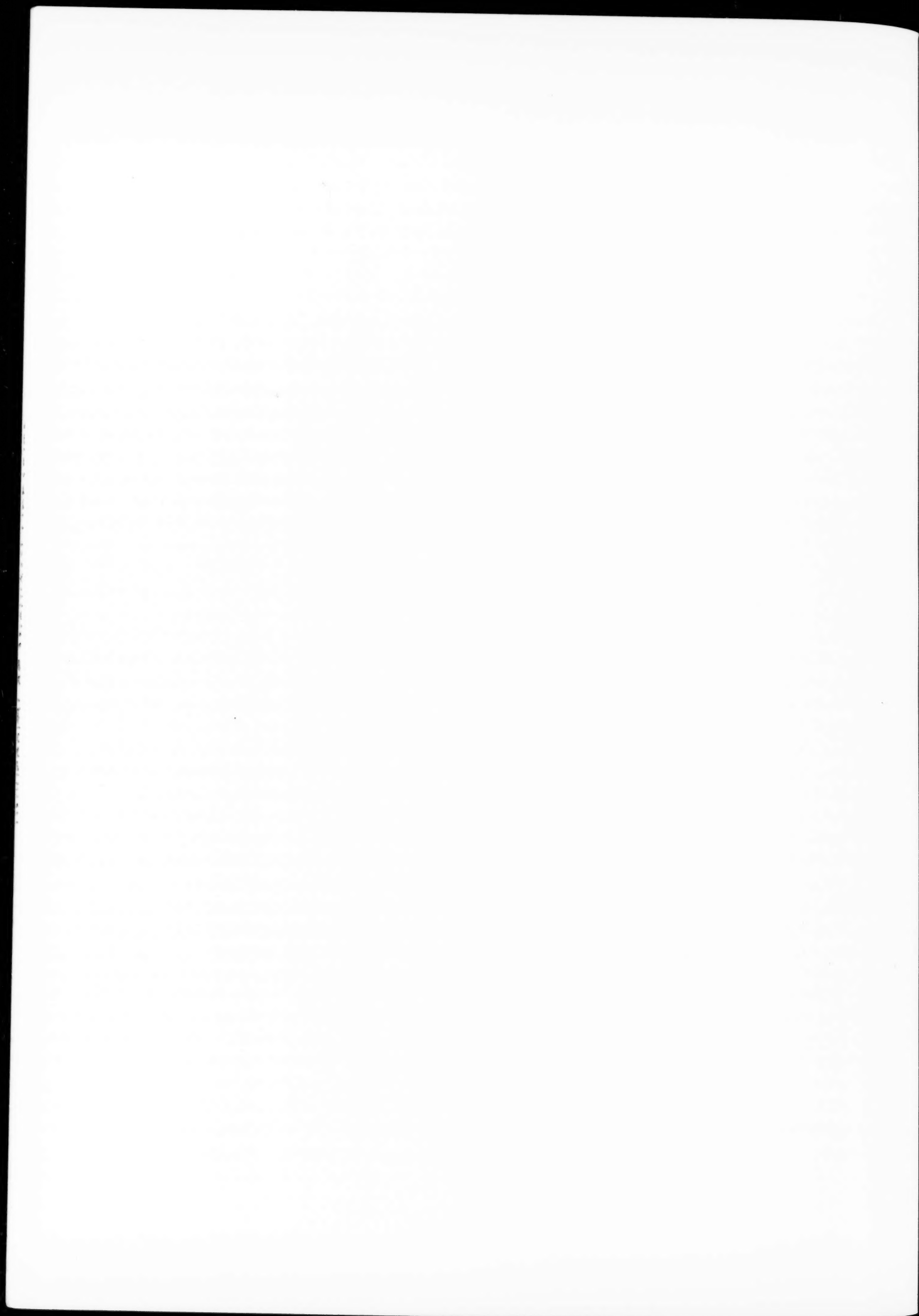
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FIG. 1.—Typical liver section from mice fed 4-dimethylaminoazobenzene for 4 months. Many nuclear inclusion bodies are present; five inclusion bodies are evident in this field, and one of these nearly fills a nucleus. Hematoxylin and eosin stain.  $\times 600$ .

FIG. 2.—Mouse liver section showing the distribution of iron-containing pigment (black deposits in photograph) in the cells of animals fed the dye. Tissue stained by the method of Tirmann and Schmelzer (4).  $\times 180$ .





# The Transplantation of Tumors to the Brains of Heterologous Species\*

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The transfer of heterologous tumor tissue to the brain was reported by Shirai in 1921 (5) and confirmed by Murphy and Sturm in 1923 (4). Despite early recognition, however, the ability of the brain to support heterologous growth has not been utilized in experimental work, nor has its application to the general problems of transplantation been explored.

One phase of a study undertaken in this laboratory to determine the factors allowing heterologous growth in the anterior chamber of the eye has been a systematic investigation of other bodily regions, including the brain, with respect to transplantation reactions. The Brown-Pearce rabbit tumor has been successfully transplanted to the subcutaneous space and testicle of the mouse (1), and several mouse tumors have been grown intramuscularly in hamsters and rats; but, in general, tumors will not survive transfer to such regions in alien hosts. In contrast, the brain has proved an excellent nidus for heterologous tissues, rivaling the anterior chamber as a growth site, and in one case providing a medium for growth after consistent failure of eye transfer.

Although in individual cases special conditions inherent in the region may act to produce differences in the incidence and rate of growth, the basic factors determining the success or failure of brain transfer appear to be identical with those concerned in anterior chamber transplantation. Adult, embryonic, and cancer tissues grow on homologous transfer, while benign tumors and precancerous tissues fail to survive, and heterologous transfer is successful only in the case of embryonic tissue and cancer. The object of the present paper is to report the heterologous growth of tumors with particular reference to those of human origin. Experiments concerned with embryonic tissues will be described at a later date.

\* This investigation was supported by grants from the Jane Coffin Childs Memorial Fund for Medical Research, The American Cancer Society, and the National Cancer Institute of the National Institutes of Health, Public Health Service.

Received for publication February 28, 1951.

## MATERIALS AND METHODS

The simple procedure devised for transfer to the brain allows rapid operation and is rarely associated with infection or mortality. After nembutal anesthesia and preparation of the skin by shaving and washing, a small incision is made through the scalp, exposing the upper anterior portion of the right parietal bone. A burr hole is drilled in the bone at a point equidistant between the sagittal and parietal sutures and approximately 0.5 cm. from the bregma. Metal drilling bits, as obtained in ordinary hardware stores, are adequate for this procedure. The bore of the bit should be slightly larger than that of the inoculating trocar, and it is essential that the cutting edge be sharp. Piercing of the inner table of the diploë imparts a recognizable sensation to the drilling hand, and, with short practice, the manipulation can be stopped before the underlying brain is injured. The use of a drill is not necessary when mice are employed, and a satisfactory opening can be obtained by the manual rotation of a pointed knife blade.

The tissue is introduced through the burr hole into the brain by means of a trocar. The trocar utilized in mouse transfers is an altered 20-gauge hypodermic needle, but for larger animals an anterior chamber trocar measuring 1½ mm. in diameter is employed. The locus at which the tissue is deposited apparently does not influence the incidence of takes. Care should be exercised, however, that the transplanted fragment remains in the brain substance when the trocar is withdrawn. The operation is terminated by approximating the edges of the skin wound and sealing them with a drop of collodion.

The species employed included rabbits, guinea pigs, rats, and mice. All the mice were of the DBA strain.

## RESULTS

*Homologous transfer.*—The transplantation of the various stock tumors carried in the laboratory was readily effected in the brains of unrelated animals of the same species. The mouse tumors included MT-8 (3); C1300, a neuroblastoma origi-

nating at the Jackson Laboratory in Bar Harbor; and MT-66, a glioblastoma obtained from Dr. Harry Zimmerman at the Montefiore Hospital in New York. The single rabbit tumor used was the Brown-Pearce carcinoma. The tumor MT-8 originated in a CBA mouse, C1300 in an A strain animal, and MT-66 in a C3H mouse. All these tumors are maintained in our colony by serial intramuscular transfer in DBA mice, and brain transfers were made in this strain. The Brown-Pearce tumor is carried by serial anterior chamber transfer in rabbits, and in homologous brain experiments the breed of the recipient was always different from that of the donor.

In all cases, takes were obtained in 100 per cent of the animals, and the tumors grew rapidly to cause death in from 1 to 4 weeks. The Brown-Pearce was the most rapidly fatal tumor, and animals rarely survived more than 9 days. MT-66 grew more slowly, and its bearers occasionally lived for as long as 4 weeks.

Histologically, the noteworthy features of growth in the brain were the abundant vascular supply and the absence of necrosis. In some cases, particularly in growths of MT-8, the vascular content of the tumor was so great as to suggest a hemangioblastoma. Supporting connective tissue stroma was extremely scanty, and, aside from thin-walled blood vessels, the tumors were almost completely parenchymatous. The size attained before death was related directly to location. Spread to the midbrain was associated with short survival and small tumors, while cerebral growths often involved the greater part of a hemisphere before death occurred.

*Heterologous transfer between laboratory animals.*—The stock tumors listed above, together with the Rous chicken sarcoma, were tested for heterotransplantability to the brain, and growth was obtained in each case (Figs. 1-6).

Experiments involving the mouse tumors MT-8, MT-66, and C1300 were limited to guinea pigs. The frequency of growth was extremely high, with many transfers resulting in 100 per cent of takes, and it seems probable that the rare failures were related to errors in technic. The tumors grew rapidly and led to death in from 4 to 6 weeks. At death, the growths usually occupied the major portion of a hemisphere and were characterized by a solid medullary structure without necrosis. Occasionally, secondary growths within the brain were found resulting from rupture of the transplant into a ventricle with "seeding." Serial transfer from brain to brain was readily effected.

The Brown-Pearce tumor was successfully transferred from the rabbit's eye to the brains of

rats, mice, and guinea pigs and has been carried by serial passage in the latter two species. The incidence of takes was uniformly high, regardless of species, and the few failures were related to infection.

Growth was rapid in the mouse and rat, and adult animals rarely survived for more than 12 days. Longer survival periods, occasionally extending to 20 days, were noted when newborn mice were used. In such cases, the intracranial pressure incident to the expanding tumor was apparently relieved by the stretching of the membranous calvarium, and large growths replacing the greater part of a hemisphere were not uncommon. In adult animals, the tumors at death averaged 0.4 cm. in diameter. On section the tumors were solid, medullary, and generally free of necrosis. Growth was invasive as well as expansive, but seeding within the brain was not observed. The tumor cells were identical with those found in the rabbit, and their presence in the alien host invoked no inflammatory reaction.

The ready transplantability of the Brown-Pearce carcinoma to the guinea pig's brain is in sharp contrast to the behavior of the tumor in other bodily regions of this species. Despite numerous attempts in many sites, including the anterior chamber of the eye, transplantation has not been effected. Although tumor cells may persist for several weeks when transferred to the eye in contact with transplants of embryonic guinea pig lung, or when the animal is given large quantities of Vitamin C, proliferation is minimal and growth can only be detected by microscopic examination. Transfer to the brain, on the contrary, is successful in 100 per cent of cases and results in large, progressively growing tumors.

While transfer is almost invariably successful, the rate of growth in the guinea pig's brain is remarkably slow, and animals survive for long periods of time. A mass of sufficient size to kill is attained in other species in 1 or 2 weeks, but in the guinea pig, indicative neurological signs rarely appear before the tenth week. If the animals are killed previous to this time, gross sections of the brain may fail to show the rather typical, demarcated mass resulting from the transplantation of other tumors, and the instance may be dismissed as a failure of transfer. However, after histological staining, a surprisingly large area of involvement becomes apparent. An explanation of this paradox is found on microscopic study and concerns a highly characteristic mode of growth (Fig. 7).

Early in the course of the transplant, the tumor cells invade Virchow-Robin spaces and with con-

tinued proliferation extend in many directions from the site of inoculation. There is little evidence of expansive growth, and the intervening brain substance is not altered. The tumor may extend in this manner to involve an area of 0.5 cm. in diameter without producing sufficient distortion to render it grossly visible. Eventually, the spaces become filled with tumor cells, and with further growth and expansion the cords of tumor coalesce, replacing brain parenchyma, and a solid mass is produced. This mass is easily recognized upon sectioning of the brain and at the time of death may occupy the major portion of a cerebral hemisphere.

Death from brain damage or increased intracranial pressure usually occurs near the end of the third month after transfer. On the other hand, the cells may persist with little growth for much longer periods, and small foci of healthy tumor only slightly larger than the transplanted fragment have been found in animals killed during the fifth month. Areas of necrosis or of glial proliferation, such as might result from regression of a tumor, have not been observed.

The tumor has been transferred from brain to brain for six guinea pig generations without alteration in the growth rate or other transplantation characteristics. Attempts have been made to transfer from the brain to the eye at each passage but have been uniformly unsuccessful.

The Rous chicken sarcoma was transferred to the brains of rabbits, guinea pigs, and mice, with an incidence of takes exceeding 85 per cent. The characteristics of the transplanted tumor were similar in all species. Growth was rapid but short-lived and invariably terminated in regression within 2 weeks of transfer. The brains of animals killed on the seventh or eighth day contained solid masses of living tumor, sometimes measuring as much as 0.5 cm. in diameter, and serial transfer undertaken at this time was always successful. The tumor was carried by consecutive passage from brain to brain for six generations in all three species, and there is no reason to believe that it could not have been maintained indefinitely by this means.

On section, the tumor was characteristically mucoid in texture and easily distinguishable from brain substance. Histologically, it was identical in cellular content and architecture to growths obtained in chickens. There was no evidence of inflammatory reaction in adjacent brain tissue, and the tumor was abundantly supplied with thin-walled blood vessels (Fig. 8).

*Heterologous transfer of human cancer.*—In past experiments human cancer has been grown in

various regions of the guinea pig's body, but such growth was obtained only after the tumor had been carried for one or more generations in the eye. Direct passage from the human patient to extra-ocular sites was never achieved, although many bodily locations were repeatedly tested. In the present experiments, the two human cancers carried for stock purposes in guinea pigs' eyes proved readily transplantable to the brain, but a finding of greater significance, sharply differentiating the brain from other bodily regions, was the fact that cancer tissue derived immediately from the human patient survived and grew.

The two human cancers maintained in the laboratory to provide stock experimental material were a glioblastoma multiforme and a carcinoma of the colon. The glioblastoma was first transplanted to the guinea pig's eye in January, 1948, by Dr. Edward Krementz,<sup>1</sup> and has been carried to date by serial anterior chamber transfer. In the present study, the tumor was successfully transferred to the brains of guinea pigs, mice, rats, and rabbits and has been passed for three consecutive generations in the two former species. The intestinal carcinoma has been maintained in guinea pigs' eyes since May, 1950, and fragments of eye growths proved readily transplantable to the brain.

Transplants of the glioblastoma are of particular interest inasmuch as they represent a human tumor growing in its natural site in an experimental animal and thus offer a unique opportunity for investigation. Transfer of the tumor is almost invariably successful, and if careful technic is employed, takes occur in all the animals used. The behavior of the transplants has been studied in guinea pigs and mice, and several significant variations have been observed. The course and termination of the tumor will be followed in rats and rabbits, but up to the present all such animals have been killed at various periods after transfer to determine the presence or absence of growth.

The presence of growth in the brains of guinea pigs and mice is not associated with neurological signs until shortly before death, and animals bearing tumors 0.5 cm. in diameter may appear in perfect health. As a rule, the guinea pigs die between the 90th and 100th day after transfer, but occasionally animals have survived to the 130th day. At death the tumors are large, free of hemorrhage and necrosis, and are well demarcated from the surrounding brain substance (Fig. 9). The survival periods of mice are more irregular in extent. Several animals have died early in the second month, with tumors occupying the greater part of a

<sup>1</sup> Jane Coffin Childs Fellow.

hemisphere, while others with tumors of comparable size have survived for as long as 120 days. The majority die between the 70th and 90th days, and the average survival period, based on 56 fatal cases, has been 83 days. At autopsy the tumors are commensurate with those found in guinea pigs but are poorly demarcated. No distinct boundary can be made out, and the tumor tissue blends gradually with the surrounding brain substance.

Histologically, the growths in the guinea pig show all the characteristics of the parent tumor (Figs. 11, 12). Mitotic figures are numerous, the cells are pleomorphic, and giant forms are common. Palisading is often a pronounced feature, and capillary endothelial hyperplasia is constant. The brain tissue about the periphery of the growth is compressed, and although it contains scattered infiltrating cells, the transition from tumor to brain tissue is quite abrupt (Fig. 13). In the mouse brain the tumor is much more sarcomatous in appearance. There are abundant mitotic figures, but the cells show little variation in size or shape. Palisading and endothelial proliferation are not common. The relationship between tumor and brain tissue is in sharp contrast with that found in the guinea pig. No boundary exists, and tumor tissue extends in irregular tongues from a central solid mass into the adjacent brain (Fig. 14). The tumor projections may extend for several millimeters with a gradual decrease in their cellular content, and the intervening brain substance appears normal. Occasionally, an area of intact brain tissue is observed completely inclosed by such "cross country" growth without evidence of degenerative cellular changes, and isolated patches of tumor are rarely found without traceable connection with the main tumor mass.

The carcinoma of the colon grows slowly and produces large amounts of mucus in the anterior chamber of the eye. Brain transplants behave in a similar manner. At 100 days, the bulk of the brain tumor consists of mucus, and most of its cells are of the "signet ring" variety (Fig. 15). Animals have been held for as long as 200 days without the development of neurological signs, and no fatalities attributable to the growth have occurred. Old transplants consist almost entirely of mucus, and tumor cells are only found after careful and prolonged search.

The transfer of cancer directly from the human patient to the brain of the guinea pig or mouse has been accomplished in ten instances. Three of the tumors were ovarian in origin, three were glioblastomas, two were derived from the intestine, one was a malignant melanoma, and one was a

mammary carcinoma. With the exception of the glioblastomas, all the growths were known to have metastasized at the time of transfer, or metastases have since become evident. Concurrent transfer to the anterior chamber of the eye was successful in each instance. A further series of twelve tumors failed to grow in the brain and also failed to grow in the eye. These included five brain tumors, a hypernephroid carcinoma of the kidney, a seminoma of the testicle, a fibrosarcoma of the chest wall, and four mammary carcinomas. The patients bearing the seven bodily tumors were free of metastasis at the time of operation and are without clinical evidence of tumor at the present time.

The ovarian cancers consisted of two papillary serous cystadenocarcinomas and one pseudomucinous cystadenocarcinoma. All the tumors were transferred to guinea pig brains, and mice were employed as additional hosts for one of the papillary carcinomas. In the latter case, the mouse brain proved as good a transplantation site as the guinea pig's brain, and at autopsy 40 per cent of the animals bore tumors. Approximately the same incidence of takes obtained in the transfer of the other tumors of the group and the behavior of the transplants was similar. Growth was rapid in all instances. Several mice killed as early as the sixth day showed microscopically visible tumors, and in one guinea pig the growth was of sufficient size by the sixteenth day to allow serial transfer. Histologically, the transplants in both guinea pigs and mice were identical with the tissue used for transfer (Figs. 16, 17).

The three glioblastomas showed the usual variations in histological structure and incidence of mitotic figures common to tumors of this type. It is of interest, although no significance can be based on data derived from only three tumors, that the growth rate of the transplants was directly proportional to the mitotic index of the primary tumor. One of the tumors containing many mature astrocytes and rare mitotic figures gave rise to neurological signs in 101 days; one with an intermediate number of mitoses required 66 days, while the tumor with the highest mitotic index resulted in death in 41 days. It is also suggestive that the slowest growing transplant was derived from the only surviving patient of the group.

The histological appearance of the transplants was similar to that of the stock glioblastoma and showed the same variation associated with growth in different species—a circumscribed, pleomorphic tumor in guinea pigs and a diffuse, sarcomatous tumor in mice.

Attempts to transplant five additional brain

tumors failed. The tumors of this group consisted of two astrocytomas, two ependymomas, and one so-called hemangioblastoma. It should be emphasized that these tumors also failed to grow when transferred to the guinea pig's eye.

The two intestinal cancers successfully transplanted were a poorly organized adenocarcinoma of the sigmoid and a colloid carcinoma of the rectum. The carcinoma of the sigmoid grew rapidly to produce neurological signs in approximately 2 months, but animals killed as early as 19 days bore tumors of sufficient size to allow large scale serial transfer. The growths in the brain were invasive, and there was no evidence of inflammatory reaction. Histologically, they resembled the parent tumor, but in some areas a higher degree of organization was evident with the production of a well defined glandular pattern (Figs. 18, 19). The colloid carcinoma of the rectum produced small growths in the brain which did not give rise to neurological signs. Guinea pigs and mice killed at varying periods from the 20th to the 80th day all showed tumors of comparable size and structure. These rarely exceeded 0.25 cm. in diameter and consisted for the most part of mucoid material with widely scattered, apparently inactive cancer cells. In the great majority of cases there was no reaction in the adjacent brain, but occasional transplants were found surrounded by phagocytosing microglia.

The breast cancer was medullary in structure, and its cells were clear, resembling those found in a hypernephroma. Takes were obtained in both mice and guinea pigs, and second generation transfers were successfully effected in both species. The mice were killed at various periods before the development of signs, and it is of interest that growths of sufficient size to be visible on gross section were found as early as the twelfth day. Neurological signs became evident in guinea pigs about the 60th day, and the brains of animals killed at this time contained large tumors histologically indistinguishable from the primary growth (Figs. 10, 20, and 21). Control transplants in the anterior chamber of the eye grew very slowly and had no more than doubled in size by the time brain transplants had attained a diameter of 0.5 cm.

The malignant melanoma was derived from the skin of the back of a 2-year-old child and, contrary to the usual behavior of this type of tumor in pre-pubertal age groups, had metastasized widely. Takes were obtained in the majority of mice and guinea pigs used. The transplants grew slowly in both species, and deaths did not occur until after the 90th day. The tumors found at death, how-

ever, were the largest noted in the present series and, in several cases, had ruptured into a ventricle; and secondary growth was found in other parts of the brain (Fig. 22). Histologically, the tumors were amelanotic and resembled the parent growth in all details. As in the case of the previously described mammary cancer, brain growth was more rapid than anterior chamber growth.

#### DISCUSSION

The major disadvantage of the brain as a site for heterologous tumor transplantation is the inability of the investigator to see or to palpate the growing transplant. Neurological signs suggestive of intracranial growth do not become apparent until shortly before death, and there is nothing in the animal's behavior to indicate the presence or absence of growth. This handicap can be overcome to a certain extent by brain biopsy. The drill hole in the calvarium remains open for a long period of time, and if the biopsy needle is directed along the path taken by the trocar at transfer, specimens can be obtained with relative ease. However, this becomes a laborious procedure when many animals are involved, and in investigations concerned with quantitative measures the anterior chamber is the superior transplantation site.

On the other hand, some features of brain growth render it preferable for certain types of experimentation. The transplants grow to larger size, they are always medullary with a minimum of connective tissue stroma, and, representing the tumor in almost pure culture, offer a unique material for chemical or immunological study. From a more particular point of view, the ability to grow human brain tumors in the brains of laboratory animals offers an opportunity for the investigation of a highly fatal and poorly understood group of human cancers under conditions closely approaching their natural state.

The fact that human cancer will survive and grow in the brain substance of mice is of significance from both biological and clinical standpoints. In previous experiments, it had been found that the mouse eye was far inferior to the guinea pig's eye as a nidus for the growth of human cancer, but in contrast it offered a much better medium for the growth of rabbit cancers (2). Other observations in line with this finding have suggested that a grouping of species with reference to the ability to synthesize Vitamin C coincides with susceptibility or resistance to heterologous transfer. Transfer between species with the same type of C metabolism (man and guinea pig) is comparatively easy, while transfer between species with different types (man and mouse) is

difficult. The point to be emphasized in the present connection is that while these considerations pertain to the eye and other bodily regions, they do not apply to the brain. A further indication that the conditions prevailing in the brain differ from those in the rest of the body is given by the behavior of the Brown-Pearce rabbit cancer in guinea pigs, for this tumor fails to survive transfer to the eye or other bodily region yet grows readily in the brain. Whether or not the different status of the brain concerns Vitamin C metabolism or some other factor is the subject of continued study. In any case, it would appear, on a basis of transplantation reactions, that the brain substances of different animal species bear a closer relationship to each other than do other bodily tissues.

The rapidity with which human cancer grows in the mouse brain, together with the comparative low cost of mice, are factors of importance in the clinical use of heterotransplantability as a prognostic test. It has been found that with the exception of brain neoplasms, only metastasizable tu-

mors are heterotransplantable, and this fact is utilized to determine the status of human tumors with respect to metastasizability at the time of their removal. Guinea pigs are relatively expensive as regards both cost and maintenance, and in the case of certain tumors a month or more is required to determine the results of transfer. It is suggested that the mouse brain may be used in place of the guinea pig's eye with advantage from the standpoint of both time and economy.

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FIG. 1.—Cross section of brain of mouse bearing transplant of Brown-Pearce rabbit tumor. Mouse was killed 7 days after transfer.  $\times 7$ . (All sections stained with hematoxylin and eosin.)

FIG. 2.—Cross section of brain of guinea pig bearing transplant of MT-8, mouse tumor, 20 days after transfer.  $\times 3$ .

FIG. 3.—Cross section of brain of guinea pig bearing transplant of C1300 mouse tumor 20 days after transfer.  $\times 3$ .

FIG. 4.—Cross section of brain of guinea pig bearing transplant of MT-66 mouse tumor 33 days after transfer.  $\times 3$ .

FIG. 5.—Cross section of brain of guinea pig bearing transplant of Brown-Pearce rabbit tumor 32 days after transfer.  $\times 3$ .

FIG. 6.—Cross section of brain of rabbit bearing transplant of Rous chicken sarcoma 10 days after transfer.  $\times 7$ .

FIG. 7.—Section of Brown-Pearce rabbit tumor growing in the brain of a guinea pig. Note extension along Virchow-Robin's spaces.  $\times 35$ .

FIG. 8.—Section of Rous chicken sarcoma growing in guinea pig's brain. Photograph was taken at junction of tumor and brain substance to show the absence of an inflammatory reaction.  $\times 100$ .



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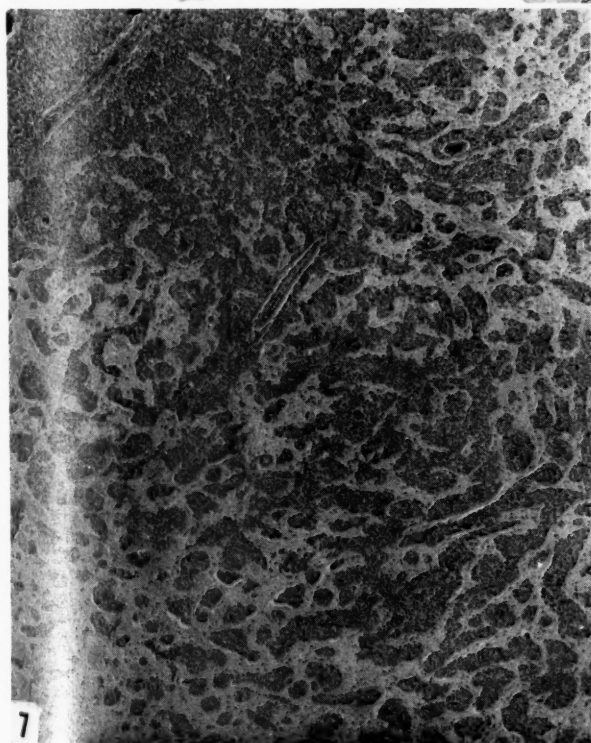
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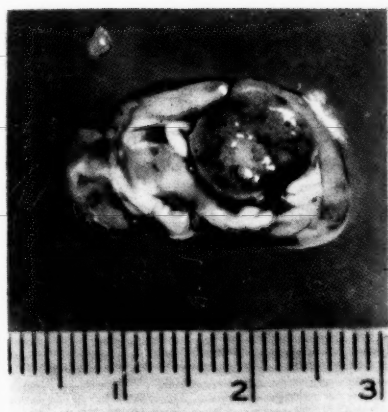
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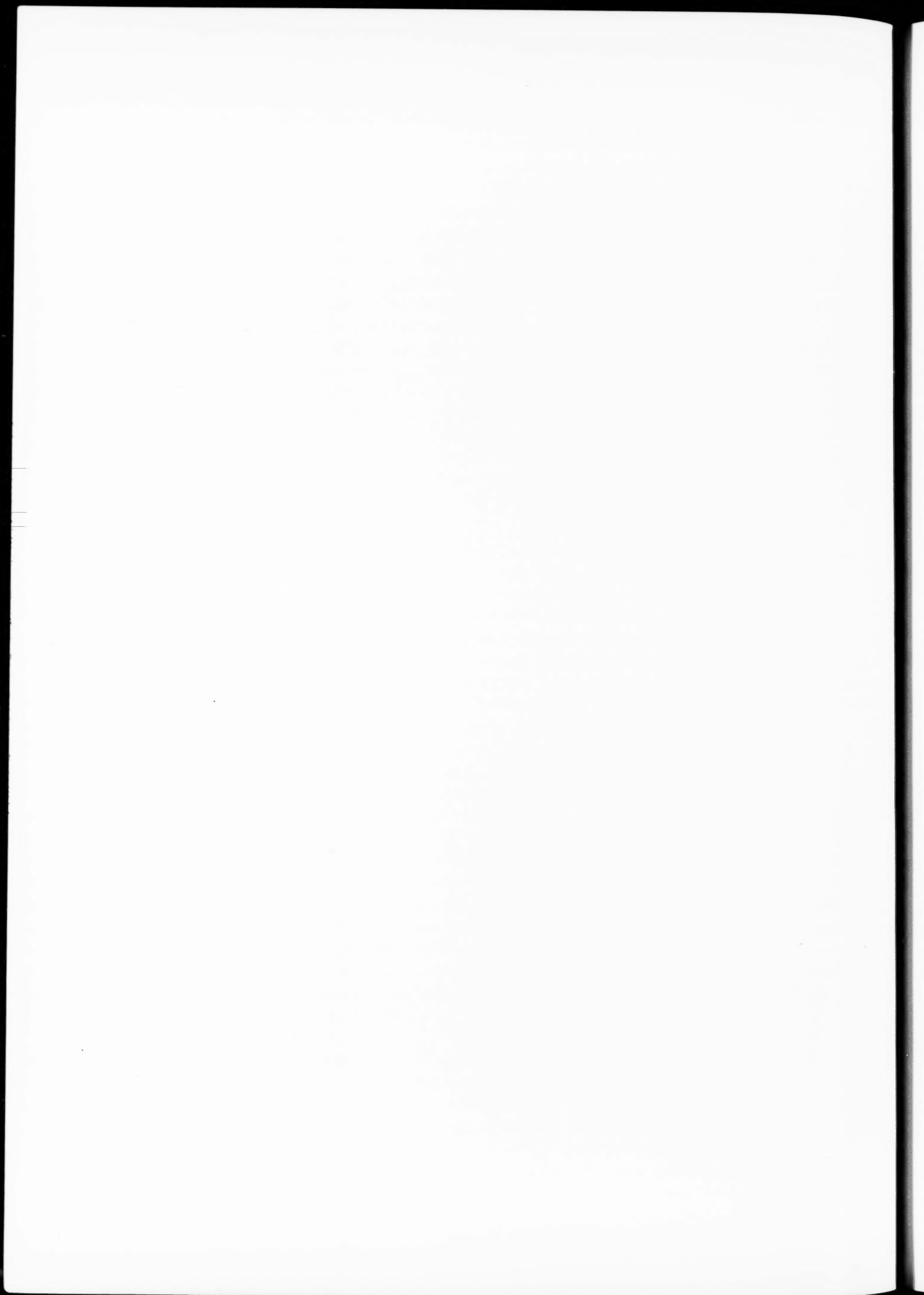


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FIG. 9 (*left*).—Cut surface of brain of guinea pig bearing growth of a human glioblastoma multiforme. The animal was killed 94 days after transfer.

FIG. 10 (*right*).—Cut surface of brain of guinea pig bearing growth of a human mammary carcinoma. The animal was killed 60 days after transfer.





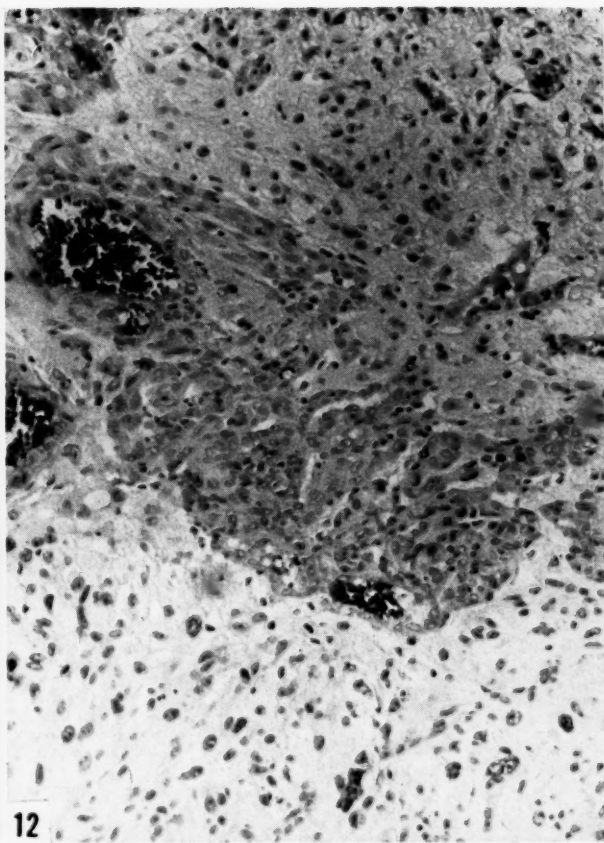
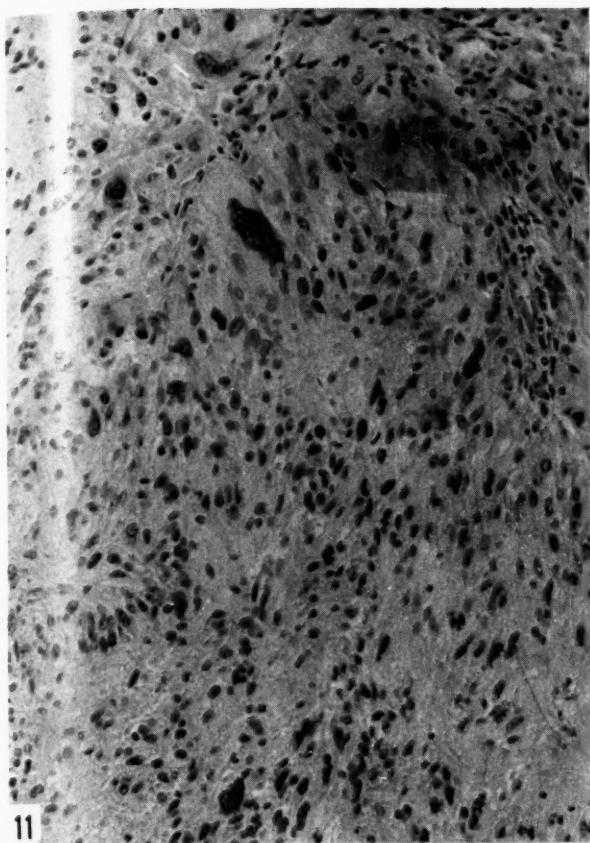


FIG. 11.—Section of transplant of human glioblastoma in guinea pig's brain.  $\times 200$ .

FIG. 12.—Section of transplant of human glioblastoma in guinea pig's brain showing the characteristic capillary endothelial hyperplasia.  $\times 200$ .

FIG. 13.—Human glioblastoma growing in guinea pig's brain. Section taken at edge of tumor to show the relatively abrupt boundary between tumor and adjacent brain. Compare with following figure.  $\times 200$ .

FIG. 14.—Human glioblastoma growing in mouse brain. There is no distinct boundary between tumor and brain substance. The tumor is infiltrating white substance between rows of ganglion cells for a considerable distance beyond main tumor mass.  $\times 200$ .

FIG. 15.—Section of guinea pig's brain bearing a transplant of a carcinoma of a human colon. Note signet ring cells and abundance of mucus.  $\times 260$ .

FIG. 16.—Section of guinea pig's brain bearing a transplant of a human ovarian papillary serous cystadenocarcinoma. The animal was killed 10 days after transfer.  $\times 200$ .

FIG. 17.—Section of mouse brain bearing a transplant of a serous cystadenocarcinoma derived from the ovary of a different human patient. The mouse was killed 6 days after transfer.  $\times 100$ .

FIG. 18.—Cross section of guinea pig brain bearing a transplant of a carcinoma of a human sigmoid colon. The animal was killed 57 days after transfer.  $\times 5$ .

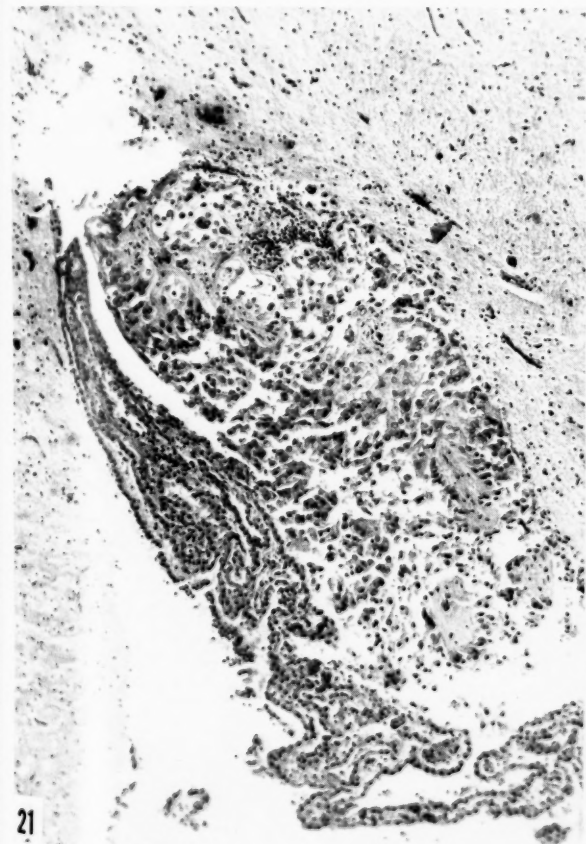
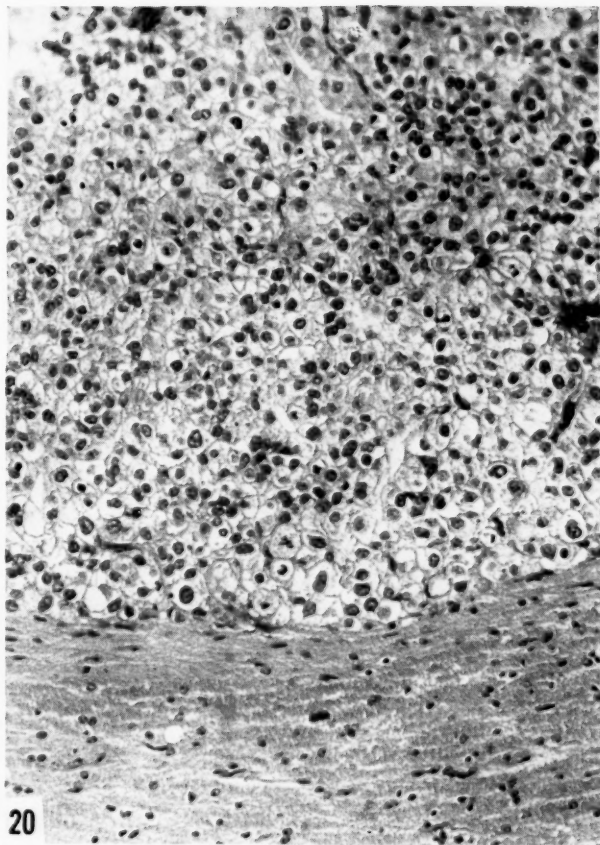
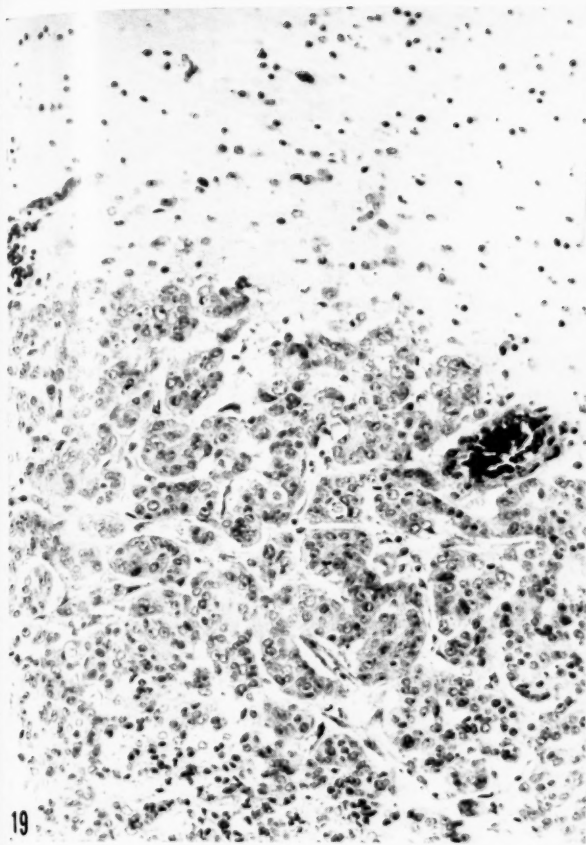


FIG. 19.—Higher power view of tumor shown in Fig. 21.  $\times 200$ .

FIG. 20.—Section of guinea pig brain bearing a transplant of a human mammary carcinoma. This is a magnification of the tumor shown in Fig. 10.  $\times 190$ .

FIG. 21.—Section of mouse brain bearing transplant of human mammary carcinoma. The mouse was killed 8 days after transfer.  $\times 100$ .

FIG. 22.—Section of a guinea pig's brain bearing a transplant of a human malignant melanoma. Section shows growth of a secondary tumor in ventricular wall as a result of "seeding" from the main mass. The animal was killed 90 days after transfer.  $\times 35$ .



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# Neoplasms in Rats Treated with Pituitary Growth Hormone

## V. Absence of Neoplasms in Hypophysectomized Rats\*

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The multiplicity and diversity of neoplasms in normal adult female rats injected for long periods with pituitary growth hormone have been previously reported (2-5). This paper is concerned with the absence of such tumors in hypophysectomized rats similarly treated with growth hormone.

A group of adult female rats of the Long-Evans strain, 148-237 days of age, was hypophysectomized. These animals were observed for a period of 2 weeks in order to determine the completeness of hypophysectomy. From these rats an experimental and a control group were selected.

Physico-chemically pure pituitary growth hormone (1) was given to a group of fifteen female rats daily for 6 days a week. The initial daily dosage of growth hormone was 0.4 mg.; this was increased to a maximum of 2.5 mg. One animal died shortly after the beginning of the experiment. Seven of the rats were sacrificed 305-346 days after growth hormone injections were begun because of their poor condition. The remaining seven experimental rats were killed after 391 and 392 days of injection. (This period of injection was less than the maximum period of injection of 485 days used in the normal rats but was greater than the minimum period of 350 days.)

The control group of fifteen hypophysectomized rats received human serum albumin. It was necessary to sacrifice three of these rats after 271-333 days of injection. The remainder was sacrificed 392 days after the first injection.

At autopsy the organs were examined under dissecting binocular microscopes. The completeness of the hypophysectomy was confirmed by examination of the sella turcica. Representative sections of all organs were taken for microscopic

study. The thyroid glands, adrenal glands, and ovaries were sectioned serially.

The controls showed no significant change in body weight. The experimental animals receiving growth hormone doubled their body weight and greatly exceeded the length of the controls (Chart 1 and Tables 1 and 2). This response was similar to that of a previous group of hypophysectomized rats that were so treated (6). The findings in the various organs are given below.

### LUNGS

*Control rats.*—The lungs were remarkably free from infection. Bronchiectasis and squamous metaplasia of the bronchial epithelium occurred in one. Bronchopneumonia occurred in three other rats (Table 3). The peribronchial lymphoid tissue was not increased in amount in any of the controls. No tumors were present.

*Experimental rats.*—Hyperplasia of the peribronchial lymphoid tissue was observed in three rats. None had pulmonary lymphosarcoma. These findings were in sharp contrast to the universal occurrence of peribronchial lymphoid hyperplasia and frequent occurrence of lymphosarcoma in rats possessing an intact pituitary when injected with growth hormone (3). Bronchiectasis with bronchopneumonia was present in three of the rats. Bronchopneumonia was present in an additional four rats (Table 4).

### LYMPHATIC TISSUES

*Controls.*—The cervical, mediastinal, and abdominal lymph nodes were usually less than 1 mm. in their maximum dimension and frequently barely visible to the naked eye. Microscopically, the lymphoid follicles were small, and germinal centers were not evident.

The spleens of the control hypophysectomized rats varied from 306 to 641 mg. The Malpighian corpuscles were small; no germinal centers were present.

The thymus glands were completely involuted

\* Aided by grants from the Public Health Service RG-409 and C421; the Research Board of the University of California; the American Cancer Society, Inc., N.Y.; and the University of California Cancer Grant.

Received for publication March 7, 1951.

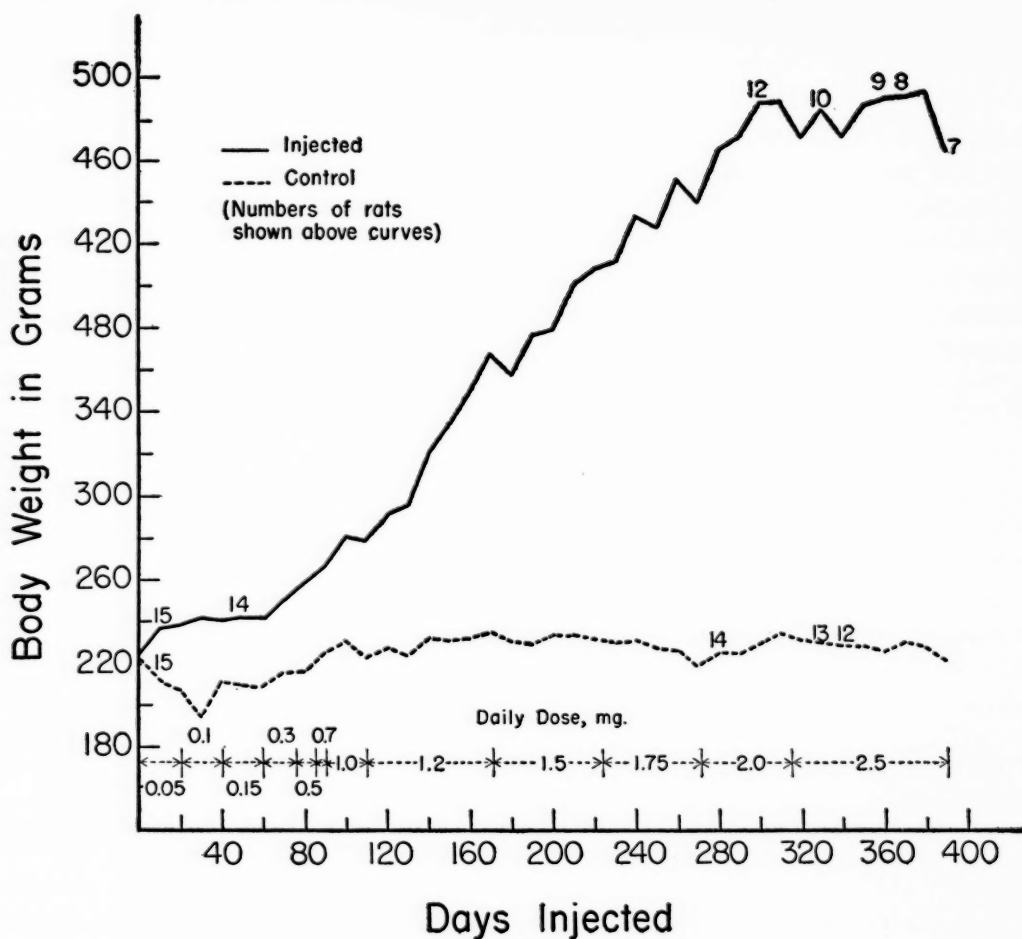


CHART 1.—Curves showing the body weights of hypophysectomized adult female rats injected over long periods of time with pituitary growth hormone and their protein-injected controls.

TABLE 1  
BODY WEIGHT AND LENGTH OF HYPOPHYSECTOMIZED FEMALE CONTROLS  
INJECTED WITH SERUM ALBUMIN

ANIMAL No.	AGE OF AUTOPSY (DAYS)	DAYS INJECTED	BODY WEIGHT (GM.)		Gain	LENGTH AT AUTOPSY (CM.)
			Start, 14 days post- operative	Autopsy		
B5147	432	271	194	189	- 5	35.5
BH4585	491	326	210	230	20	37.4
B1089	555	333	250	239	-11	37.0
B9307	641	392	272	245	-27	40.4
B9309	641	392	234	211	-23	38.2
G1112	614	392	221	273	52	38.5
GH1118	614	392	205	199	- 6	36.0
W1119	614	392	235	218	-17	36.8
G6827	687	392	213	203	-10	36.5
B4535	560	392	249	221	-28	38.7
B1041	615	392	216	190	-26	36.5
GH4550	560	392	190	186	- 4	
G4559	560	392	221	250	29	38.0
B5174	553	392	219	229	10	36.8
G1099	614	392	207	232	25	38.3
AV. 12*	606		223	221	- 2	37.7

\* Only data from rats injected with serum albumin for 392 days are included in the averages.

in ten rats. In three rats the weights varied from 72 to 140 mg.

*Experimental rats.*—The cervical, mediastinal, and abdominal lymph nodes were larger than those in the controls, but none of the lymph nodes were larger than 0.3 cm. in maximum dimension. The follicles were visible macroscopically. There were no lymphosarcomas.

The spleens of the hypophysectomized rats injected with growth hormone were greatly enlarged and varied from 630 to 2,380 mg. The Malpighian corpuscles were larger than those seen in the controls.

The thymus glands varied greatly in size. In four rats they were completely atrophied, and in ten rats the weights ranged from 66 to 271 mg.

#### ADRENAL GLANDS

*Controls.*—The adrenal glands of the hypophysectomized controls injected with albumin were very small and had a dull gray-yellow appearance. The weights ranged from 10 to 26 mg., with an average weight of 19 mg. Histologically, the adrenal cortices showed the atrophy typical of hypophysectomized rats. In one rat there was nodularity of the cortex. The medullae were com-

TABLE 2  
BODY WEIGHT AND LENGTH OF HYPOPHYSECTOMIZED FEMALE RATS  
INJECTED WITH PITUITARY GROWTH HORMONE

ANIMAL NO.	AGE AT AUTOPSY (DAYS)	DAYS INJECTED	BODY WEIGHT (GM.)			LENGTH AT AUTOPSY (CM.)
			Start, 14 days post- operative	Autopsy	Gain	
GH5170	453	292	185	421	235	40.9
G2429	488	298	198	417	219	44.2
G1148	544	322	252	480	230	43.9
B2469	511	322	220	369	149	41.5
B1091	573	351	210	452	242	42.4
B2528	550	361	216	430	214	45.9
G1161	604	382	237	579	342	50.7
BH2500	580	391	251	531	280	45.5
B1132	614	392	213	437	224	43.0
G1142	613	391	243	435	192	43.9
B1073	614	391	270	527	257	48.1
B4583	559	391	199	289	90	39.2
GH2494	580	391	207	581	374	47.0
W9295	641	391	225	506	281	43.4
AV. 7*	600		230	473	243	44.3

\* Only data from rats injected with growth hormone for 391–92 days are included in averages.

TABLE 3  
ORGANS OF HYPOPHYSECTOMIZED CONTROLS INJECTED WITH SERUM ALBUMIN

ANIMAL NO.	LUNGS	THYMUS (mg.)	SPLEEN (mg.)	Weight (mg.)	ADRENAL GLANDS		OVARIES
					Cortex	Medulla	
B5147					Atrophic	Normal*	
BH4585			375	14	Atrophic	Normal	
B1089		Atrophic			Atrophic	Normal	
B9307		Atrophic	542	21	Atrophic	Normal	
B8309		Atrophic	398	16	Atrophic	Normal	
G1112		Atrophic	590	16	Atrophic	Normal	
GH1118	Pneumonia	Atrophic	306	10	Atrophic	Normal	
W1119	Pneumonia	140	400	24	Atrophic	Normal	Atypical interstitial tissue
G6827	Pneumonia	Atrophic	307	20	Atrophic	Normal	Tumor
B4535	Bronchiectasis, pneumonia	Atrophic	462	22	Atrophic	Normal	
B1041		Atrophic	345	15	Atrophic	Normal	
GH4550		98	492	16	Atrophic	Normal	
G4559	Pneumonia	72	641	23	Atrophic	Normal	
B5174		Atrophic	484	26	Atrophic	Normal	Atypical interstitial tissue
G1099		Atrophic	362		Nodular	Normal	
AV. †			444(12)	19(11)			

\* Or slightly hypoplastic.

† The averages are based only on the animals sacrificed after 392 days of injection. The number of animals is shown in parentheses following the average figure.

posed of polyhedral, flat, and crescentic cells with less abundant cytoplasm and smaller nuclei than in normal rats. There were no tumors of the medulla.

*Experimental Rats.*—The adrenal glands of the hypophysectomized rats injected with growth hormone were larger than those of the controls. The combined weights of the right and left adrenal glands varied from 18 to 39 mg., with an average weight of 28 mg. There was nodularity of the adrenal cortices in nine of the fourteen rats. The width of the cortex and the lipoid distribution were similar to those noted in the controls. The medullae were larger than in the controls and consisted

by the neoplastic cells.

The uteri were atrophic, and the weights varied from 56 to 170 mg. The endometrium consisted of a few small glands and atrophic stroma; there were no neoplasms. The mammary glands were atrophic in all controls; no tumors were present.

*Experimental rats.*—The ovaries of the hypophysectomized rats injected with growth hormone were larger than those of controls. The right and left ovaries of the experimental rats had an average combined weight of 39 mg., as compared to an average weight of 15 mg. for the controls. The follicles were small. The interstitial tissue was fre-

TABLE 4  
ORGANS OF HYPOPHYSECTOMIZED RATS INJECTED WITH PITUITARY GROWTH HORMONE

ANIMAL NO.	LUNGS	THYMUS (mg.)	SPLEEN (mg.)	Weight (mg.)	ADRENAL GLANDS		OVARIES
					Cortex	Medulla	
GH5170	Pneumonia	162	1,018	24	Atrophic	Hypertrophied	
G2429		123	1,685	32	Nodular	Hypertrophied	
G1148	Bronchiectasis, pneumonia	180	1,045	24	Atrophic	Hypertrophied	Atypical interstitial tissue
B2469		82	930	33	Nodular	Hypertrophied	Atypical interstitial tissue
B1091	Pneumonia	Atrophic	1,039	32	Nodular	Hypertrophied	Atypical interstitial tissue
B2528		Atrophic	1,034	31	Nodular	Hypertrophied	Atypical interstitial tissue
G1161	Pneumonia	271	2,380	36	Atrophic	Hypertrophied	
B2500	Pneumonia	87	855	32	Nodular	Hypertrophied	Atypical interstitial tissue
B1132		Atrophic	1,140	39	Atrophic	Hypertrophied	Tumor
G1142		60	1,436	18	Nodular	Hypertrophied	
B1073	Bronchiectasis, pneumonia	177	1,557	30	Atrophic	Hypertrophied	
B4583		Atrophic	630	18	Nodular	Hypertrophied	
GH2494	Bronchiectasis, pneumonia	66	1,462	28	Nodular	Hypertrophied	
W9295		71	1,044	29	Nodular	Hypertrophied	
AV.*			1,161(7)	28(7)			

\*The averages are based only on the animals sacrificed after 391-92 days of injection. The number of animals is shown in parentheses following the average figure.

of cells with moderately abundant cytoplasm. Rare mitoses were observed. No medullary tumors were present.

#### REPRODUCTIVE ORGANS

*Controls.*—The ovaries with but three exceptions were small and contained only small follicles, small "deficient" interstitial cells, old corpora lutea, and a few small pseudo-testicular tubules. In two rats the ovaries contained small, irregular areas of cords and tubules derived from interstitial cells. Occasional small follicles with irregular outlines were present. In one rat (G6827) the right ovary had been replaced almost completely by a tumor. The tumor was composed of cells which were apparently derived from the theca interna or interstitial cells. These cells varied in shape; the cytoplasm was scanty; the nuclei were round or oval and vesicular. Mitoses were frequent. These cells were frequently arranged in compact masses and occasionally into slender tubular structures and cords. A few small follicles were surrounded

quently more abundant than in the controls. The corpora lutea were similar to those of the controls. Pseudo-testicular tubules were present and were larger than in the controls. In five rats there were small areas of cords and tubules derived from interstitial cells. These areas were similar in appearance to those observed in the controls. In one rat (B1132) there was a tumor of the right ovary which had a pattern similar to that in a control (G6827); however, this tumor was much larger, and the individual cells were larger.

The uteri of the rats injected with growth hormone were much larger than those of the controls. The weights varied from 170 to 411 mg. The increase was due to cellular hypertrophy of all layers; no tumors were present. The mammary tissue showed no evidence of specific stimulation; there were no neoplasms.

#### DISCUSSION

There was almost complete absence of neoplasms in hypophysectomized rats injected with

growth hormone. This finding was in striking contrast to the occurrence of many different neoplasms in the intact rats which were similarly treated (3-5). This indicates that the presence of the pituitary gland and, hence, its secretions are necessary in the production of the neoplasms resulting from the administration of growth hormone. The long continued administration of growth hormone has been shown to produce morphologic alterations in the various component cells of the anterior pituitary (2). It appears likely that there are corresponding alterations in its functions. These findings indicate that large amounts of growth hormone and the altered physiology of the anterior pituitary are two important factors in the occurrence of neoplasms.

The lymph nodes, spleens, and thymus glands of the hypophysectomized rats were larger than those of the controls and showed evidence of specific stimulation by pituitary growth hormone. Germinal centers in the spleen were frequently seen in the rats receiving growth hormone. Peribronchial lymphoid hyperplasia, which was a universal finding in intact rats receiving growth hormone and was frequent and in frequent association with lymphosarcoma of the lungs, was not observed in the hypophysectomized rats given growth hormone.

The adrenal glands were larger in the hypophysectomized rats injected with growth hormone than in the controls. This increase in size was due to hyperplasia and hypertrophy of the medullary cells and increased vascularity of the medulla. This finding was similar to the observation previously reported for hypophysectomized rats treated with growth hormone (6). On the other hand, medullary tumors which were present in most of the normal rats injected with growth hormone (4) did not occur in the hypophysectomized rats.

There was no difference in the incidence of ovarian tumors in the experimental and control groups. In both groups transformation of interstitial tissue into cords and tubules was noted; this occurred more often in the rats injected with growth hormone. It is problematic whether such transformations without evidence of extensive

growth of the tissue in question fall into the category of neoplasia or metaplasia.

#### SUMMARY AND CONCLUSIONS

1. Long continued administration of pituitary growth hormone to hypophysectomized adult female rats, while producing continuous body growth, did not produce the numerous neoplasms that developed in rats with intact pituitary glands which were similarly treated.

2. The lymph nodes, thymus glands, and spleens were relatively and absolutely larger in the hypophysectomized rats receiving growth hormone than in the controls receiving ablumin. On the other hand, the peribronchial lymphoid tissue did not show the hyperplasia previously noted in rats with intact pituitary glands when treated with growth hormone.

3. The adrenal medullae of the hypophysectomized rats injected with growth hormone were larger than in the hypophysectomized controls. There were no medullary tumors.

4. The ovaries of the animals receiving growth hormone were larger than the ovaries of controls. A tumor of the ovary was present in one rat receiving growth hormone and in one control hypophysectomized rat. These were the only neoplasms occurring in either the experimental rats or their controls.

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# Heterologous Growth and Passages of Mouse Sarcomas in Hamsters (*Mesocricetus auratus*)\*

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It has been apparent since early in the history of cancer research that the study and experimental therapy of human cancer might be considerably advanced if the neoplasms could be grown outside of the body of the cancer patient. Besides tissue culture *in vitro*, there is the possibility of growth of tumor transplants in host animals. Experimentally, this problem resolves itself into that of the successful heterologous growth of tumors of any species. Early efforts in this field have been reviewed in a previous paper (9), which dealt with the successful heterologous growth of mouse Sarcoma 180 in suckling rats, but not in adult rats. The present communication concerns the use of hamsters as host animals for mouse sarcomas.

The hamster is a comparative newcomer to cancer research, and there are not many reports of its use.

There is a single report of spontaneous transmissible tumors in hamsters. Ashbell (1) found thirteen tumors in a total of 1,000 animals. Two polymorphous-cell sarcomas, one carcinoma, and two of ten cortical hypernephromas were transplantable to other hamsters.

The earliest experimental work reported is that of Gye and Foulds (5). A mixed-cell sarcoma transplantable to other hamsters was induced in a male injected with 3,4-benzpyrene.

Halberstaedter (6) induced a similar sarcoma with benzpyrene in a hamster. The original host had no metastases, but in the passages the tumor was occasionally found to involve lymph nodes, kidneys, and lungs.

Crabb (3) induced transplantable sarcomas in hamsters with 9,10-dimethyl-1,2-benzanthracene. Metastases occurred in lymph nodes, kidneys, and lungs in the course of seventeen passages. Attempted transplantations of the hamster sarcoma to mice failed.

Nettleship and Smith (8) transplanted a hamster fibrosarcoma induced with 20-methylcho-

lanthrene for five generations without noting any metastases.

Lutz, Fulton, Patt, and Handler (7) used the cheek pouch of the hamster as a site for the transplantation of hamster tumors.

Greene (4) transplanted the Brown-Pearce tumor of the rabbit into testicles, anterior chamber of the eye, and subcutaneous tissue of hamsters. The tumor growth that occurred was followed by regression.

## MATERIALS AND METHODS

A study was made of the ability of Crocker mouse Sarcoma 180 and a transplantable methylcholanthrene-induced spindle-cell mouse sarcoma to grow in young and adult hamsters. The latter tumor originated in 1948 in a C57 black mouse and proved to be transplantable to various strains of mice. A Swiss mouse bearing the 74th passage of this tumor was used as donor for the hamster experiments.

Cell suspensions were made by mincing a 7-day-old tumor with fine scissors and adding equal parts of Locke-Ringer solution at pH 7.0. The suspension was then injected intraperitoneally or subcutaneously in the volumes given in Tables 1 and 2.

The recipient hosts for mouse Sarcoma 180 were hamsters of various ages, but only adult hamsters were hosts for the methylcholanthrene-induced mouse sarcoma. The hamsters were from various sources over several years, but all were Syrian hamsters, *Mesocricetus auratus*.

The viability of each mouse tumor used in these hamster experiments was checked by simultaneous implantation into Swiss mice of Carworth or Rockland Farms stock. This was true not only of the mouse tumors taken from mouse hosts but also of the mouse tumors taken from hamster hosts.

## RESULTS

Results of the injection of Sarcoma 180 cell suspension into hamsters are given in Table 1. Minced Sarcoma 180 proliferated readily on injection into hamsters 1-6 days old. Hamsters injected intraperitoneally when less than 1 day old died from the

\* This work was supported in part by an institutional grant from the American Cancer Society and by a grant from the Damon Runyon Memorial Fund.

Received for publication March 8, 1951.

tumor growth within 5–12 days, the average being 9 days. Successful transplantations of the mouse tumor appeared to decrease in relative frequency with the age of the hamster hosts, although serial passages could be made in adult hamsters. Subcutaneous injections of Sarcoma 180 cells into a few hamsters 1 and 2 days old were followed by successful growth for 12–15 days before regression occurred. There were no regressions except in the four animals of experiment 177. All checks on viability of Sarcoma 180 donor tumors by injection

into mice resulted in successful tumor growth in the inoculated mice.

At autopsy of host hamsters that had received intraperitoneal injections, tumors were found in the peritoneal cavity as follows: under the liver lobes, attached midway on the spleen, on the small intestine, and along the lower gastrointestinal tract. There were also a few metastases to the lung; similar lung metastases had been obtained with Sarcoma 180 in young rats (9). Figure 1 illustrates intraperitoneal tumors of mouse Sarcoma

TABLE 1  
RESULTS WITH CROCKER MOUSE SARCOMA 180 IN YOUNG AND ADULT HAMSTERS\*

Exp. no.	ANIMALS		INJECTION		DAY HAMSTER DIED (D) OR WAS KILLED (K)		
	Age	No.	Route	cc.	TAKES	With tumor	Without tumor
					First passage, mouse to hamsters		
126	1 day	2	Subc.	0.05	2/2	K7, 7	
177	2 days	4	Subc.	0.1	4/4†		K18, 18, 18, 18
105	1 day	7	I.P.	0.05	7/7	D10, 10, 11, D12, 12, 12, 12	
126	1 day	7	I.P.	0.05	7/7	D8, 8, 8, 10; K8, 11, 11	
145	1 day	5	I.P.	0.05	5/5	D5, 6, 6; K4, 6	
155	1 day	5	I.P.	0.05	5/5	D8, 9, 9, 9, 10	
171	1 day	4	I.P.	0.03	4/4	K11, 11, 11, 15	
172	6 days	5	I.P.	0.03	4/5	K11, 11, 11, 11	K15
173	19 days	3	I.P.	0.05	1/3	K15	K11, 15
174	45 days	3	I.P.	0.05	0/3		K11, 15, 15
162	3-6 mo.	15	I.P.	0.05	2/15	K11, 11	K11, 11, 11, 11, 34, 34, 34, K34, 34, 34, 34, 34, 34
163	3-6 mo.	14	I.P.	0.05	5/14	K11, 11, 11, 34; D31	K11, 11, 11, 34, 34, 34, K34, 34, 34
168	3-6 mo.	15	I.P.	0.05	1/15	K21†	K21 (14 hamsters)
Second passage, hamster to hamsters							
169	4-7 mo.	5	I.P.	0.05	1/5	K17†	K17, 17, 17, 17
Third passage, hamster to hamsters							
175	4-7 mo.	3	I.P.	0.1	1/3	K25†	K25, 25
	4-7 mo.	2	Subc.	0.1	0/2		K25, 25
Fourth passage, hamster to mice (The sarcoma grew in all mice injected.)							

\* Accompanying the passages into hamsters, control transplantations into mice always grew. I.P. denotes intraperitoneal, Subc. denotes subcutaneous inoculation.

† Only the sarcomas in the four hamsters of experiment 177 regressed, after an initial period of growth.

‡ Donor for next passage.

TABLE 2  
RESULTS WITH METHYLCHOLANTHRENE-INDUCED MOUSE SARCOMA IN ADULT HAMSTERS\*

Exp. No.	ANIMALS		INJECTION		DAY HAMSTER KILLED (K) OR FOUND DEAD (D)		
	Age (mo.)	No.	Route	cc.	TAKES	With tumor	Without tumor
	First passage, mouse to hamsters						
164	3-6	13	I.P.	0.1	4/13	K11, 11, 21, 21†	K11, 11, 11, 11, K21, 21, 21, 21, 21
165	3-6	12	I.P.	0.1	1/12	K21	K11, 11, 11, 11, 11, 11, K21, 21, 21, 21, 21
Second passage, hamster to hamsters							
170	4-7	5	I.P.	0.1	4/5	D13; K16, 16, 16†	K16
Third passage, hamster to hamsters							
176	4-7	2	Subc.	0.1	0/2		K25, 25
	4-7	3	I.P.	0.1	3/3	K25, 25, 25†	
Fourth passage, hamster to mice (The sarcoma grew in all mice injected.)							

\* There were no regressions in those hamsters in which tumor growth occurred. In the passages above, control transplantations of the sarcoma into mice always grew. I.P. denotes intraperitoneal, Subc. denotes subcutaneous inoculation.

† Donor for next passage.

180 in an adult hamster, while Figure 2 shows them in two young hamsters. In Figure 2, line *b* indicates metastatic nodules in the lung. Figure 3 is a photomicrograph of a lung section with Sarcoma 180 metastases from a young hamster 12 days after intraperitoneal injection of Sarcoma 180 mince.

The Sarcoma 180 donor tumor used for the second passage was taken from a hamster 21 days after implantation. On histological examination,<sup>1</sup> the donor tumor showed a little necrosis, but mitoses and viable cells were present. The tumor for the third passage was 17 days old; it had a necrotic center but viable cells peripherally. The tumor for the fourth passage, 25 days old, was largely necrotic but contained some viable areas. It is to be noted that Sarcoma 180 is usually transplanted in this laboratory from mouse to mouse every 7 days, because necrosis in older tumors reduces the frequency of successful transplantation.

Results of the injections of minced methylcholanthrene-induced mouse tumor into adult hamsters are given in Table 2. In the first passage, 5 of 25 hamsters injected intraperitoneally with tumor from a mouse developed tumors. The donor tumor for the second passage was taken after 21 days' growth in a hamster; intraperitoneal inoculations gave successful tumor growth in four of five hamsters. The donor tumor for the third passage was 16 days old; all of three intraperitoneal injections, but neither of two subcutaneous injections, were followed by successful growth. The fourth passage donor tumor, 25 days old, was injected into mice only, with complete success. No regressions were noted in any of the hamster hosts of this mouse sarcoma, although they were allowed to live for 11–25 days after inoculation. Figure 4 illustrates the intraperitoneal tumors in an adult hamster injected with this mouse sarcoma. The histological appearance of the methylcholanthrene tumor in its 74th passage in mice, in the hamster, and again in the mouse, after having grown in a hamster, is illustrated in Figures 5, 6, and 7, respectively. The cells appear to have undergone no drastic alteration in morphology, despite the changes of host.

<sup>1</sup>The authors are indebted to Dr. J. D. Allen, of the Pathology Department, Memorial Hospital, for the histological examinations.

FIG. 1.—Adult hamster bearing tumors and dead 31 days after intraperitoneal injection of 0.05 cc. of Sarcoma 180 mince: *a* and *c*, tumors on intestine; *b*, tumor mass under lobe of liver; *d*, tumor mass attached to spleen. Exp. 163.

FIG. 2.—Two young hamsters (one dead, one killed) bearing tumors 6 days after intraperitoneal injection of 0.05 cc. of

## DISCUSSION

Sarcoma 180 transplants grew on inoculation into the peritoneal cavity of young rats but not in adults (9), while in hamsters this sarcoma evinced some ability to grow through successive passages in adult animals. The methylcholanthrene-induced spindle-cell sarcoma of the mouse also grew through successive intraperitoneal passages in adult hamsters. It thus appears that the hamster may prove to be a better host animal for heterologous tumors than is the rat, and it is suggested that the possibility of using the hamster as a culture medium for human neoplasms be investigated.

## SUMMARY

Serial passages of Crocker mouse sarcoma 180 and a methylcholanthrene-induced mouse sarcoma were made in adult hamsters by means of intraperitoneal inoculation. Sarcoma 180 was transplanted from the mouse to the peritoneal cavity of the hamster with greater success when the hamsters were 1 or a few days old than when older. Subcutaneous inoculations in adult hamsters failed to grow and in suckling hamsters regressed after about 2 weeks of growth. No regressions were noted with either mouse sarcoma implanted intraperitoneally in the hamster.

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Sarcoma 180 mince: *a* and *c*, tumor on liver; *b*, tumor nodules in lung; *d*, tumor mass in abdominal cavity. Exp. 145.

FIG. 3.—Sarcoma 180 metastases in lung of young hamster that died 12 days after intraperitoneal injection of 0.05 cc. of Sarcoma 180 mince. Exp. 105. Mag.  $\times 150$ .

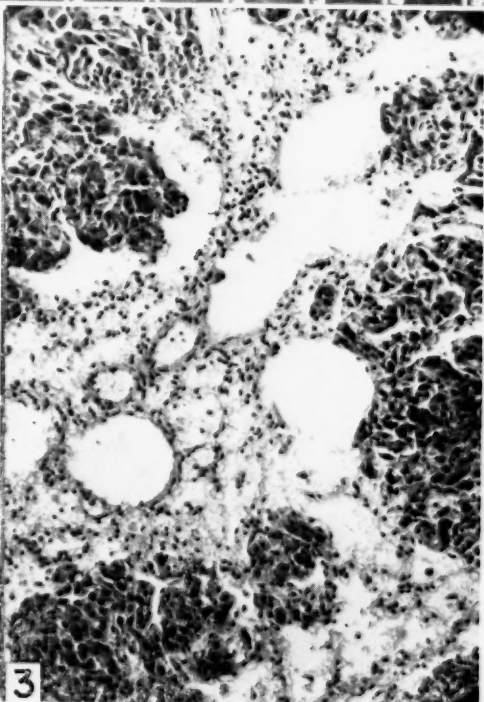
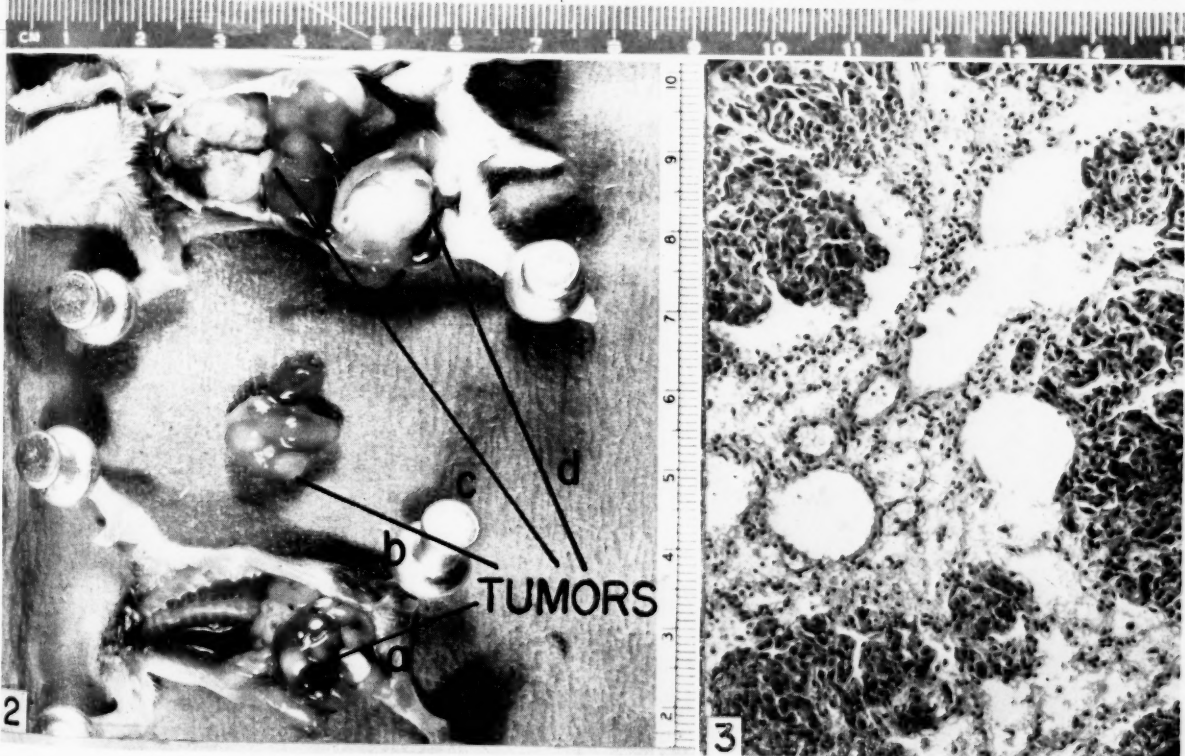
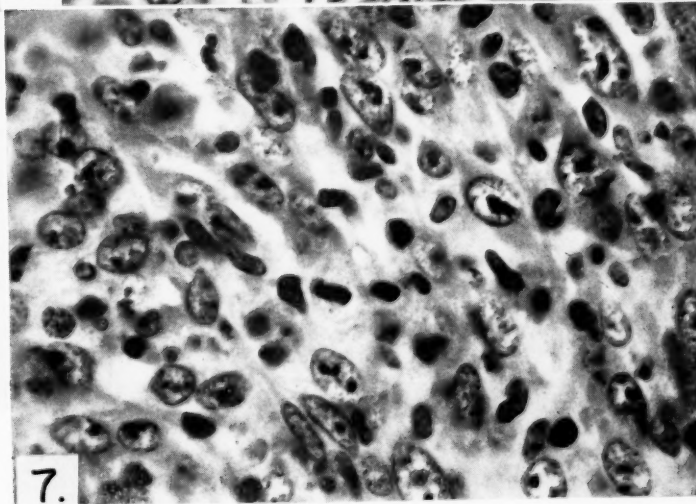
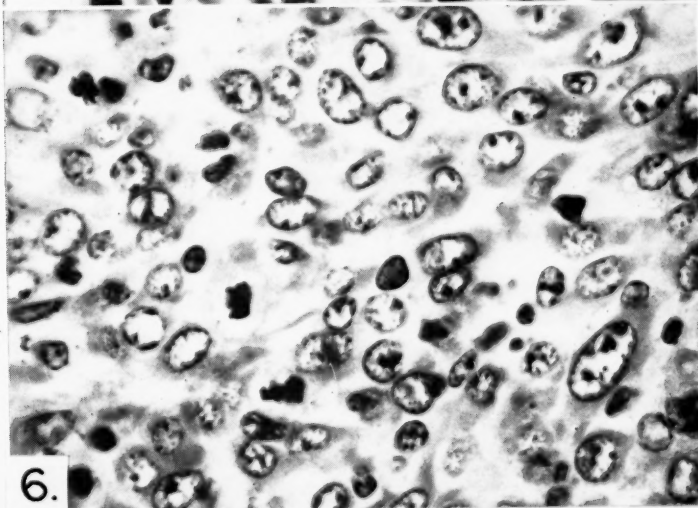
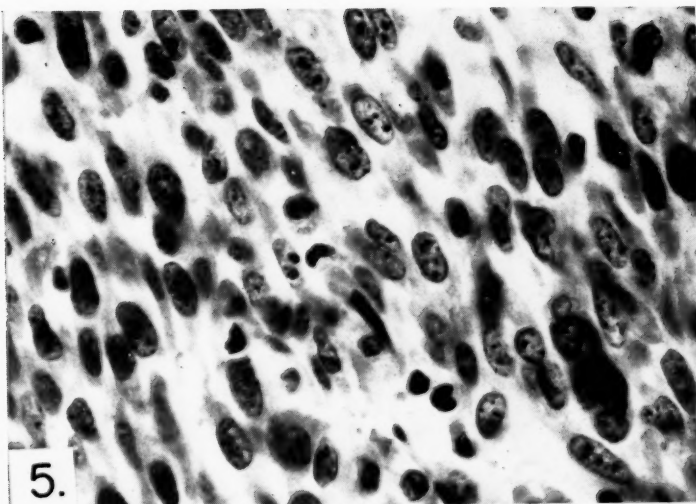


FIG. 4.—Adult hamster with intraperitoneal tumors, killed 25 days after injection of 0.1 cc. of mince of methylcholanthrene-induced mouse sarcoma: between *a* and *b*, a cluster of tumors. Exp. 176.

FIG. 5.—Methylcholanthrene-induced spindle-cell mouse sarcoma, 74th passage in mice. Zenker fixation, hematoxylin and eosin stain. Mag.  $\times 675$ .

FIG. 6.—Methylcholanthrene-induced mouse sarcoma in hamster. Zenker fixation, hematoxylin and eosin stain. Mag.  $\times 675$ .

FIG. 7.—Methylcholanthrene-induced mouse sarcoma growing in mouse after passage through hamster. Zenker fixation, hematoxylin and eosin stain. Mag.  $\times 675$ .



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# On the Specificity of Hypervolemia and Congestive Changes in Tumor-bearing Mice\*

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Ever since it was noted that estrogen-secreting granulosa-cell tumors in mice are associated with hypervolemia and with a hitherto unknown cavernous type of congestive change (5, 7, 13), data were gathered on the specificity of the relationship between granulosa tumors and hypervolemia, and experiments were undertaken to learn about homeostasis of blood volume.

Until recently, all spontaneous neoplasms associated with hypervolemia that were found were of the granulosa-cell type, and all recently studied transplanted granulosa tumors were found to cause hypervolemia (1, 2). There was, however, no consistent parallelism between the degree of the blood volume rise and estrogenization (1, 8). The hypervolemia of granulosa tumors was found to be due to a selective rise of the plasma (albumin) volume with maintenance of normal erythropoiesis (7, 8, 13). Luteomas were found to cause a mild degree of hypervolemia due to polycythemia, with an occasional slight secondary plasma volume rise and a mild hypervolemic congestion of viscera (8).

Blood volume determinations are seldom made in tumor-bearing hosts, but the congestive changes associated with hypervolemia are so characteristic that they could hardly have escaped notice. Inquiries of colleagues with wide experience in the pathology of diverse animal tumors confirmed our impression that such congestive changes are absent in animals bearing common types of neoplasms; their occurrence in mice with granulosa tumors has, however, been confirmed (2).

This is a report on several nonhormone-secreting neoplasms observed which cause some hypervolemia. A nonestrogen-secreting ovarian neoplasm (strain XIX) was found to be frequently associated with both widespread cavernous congestion and hypervolemia. Histogenetically, this tumor strain is probably related to granulosa tumors. A mild plasma volume rise without congestive changes was also found with two neo-

plasms of mammary gland origin. One of these, strain M, was found to cause a slight hypervolemia with a characteristic cavernous congestion of ovaries resembling a hemangioma. In general, extreme congestive changes and hypervolemia were encountered only in mice bearing tumors related to gonadal cells. Hypervolemia with other neoplasms, notably of the breast, is mild and is accompanied by anemia. It is possible that the rise of plasma volume with these neoplasms is secondary to anemia.

## MATERIAL AND METHODS

*Animal and tumor strains.*—The ovarian tumors were induced by x-rays in Rf/Ak hybrids. When large, the grafted tumors were transplanted to 2–4-month-old mice of this stock (cf. 3). Breast carcinoma strain M, originated in C3H mice, was obtained from Dr. Alton Meister of the National Cancer Institute and is designated by him as “adenocarcinoma C3HBA.” The breast tumor, strain R, originated in a C3H mouse. X-rayed C57 black and recipient mice were obtained from Dr. William Russell of Oak Ridge National Laboratory. The myoepithelioma and sarcoma strains were kindly sent us by Dr. Thelma B. Dunn of the National Cancer Institute. The myoepithelioma was the 24th generation of a tumor that originally arose in the stomach wall of a strain C mouse following the injection of methylcholanthrene.

Normal Rf/Ak hybrid mice served as controls for the mice bearing ovarian carcinoma XIX and granulosa tumor V. For the breast carcinoma M and breast carcinoma R, normal C3H mice served as controls. In this latter strain, there being no significant sex differences, both sexes were combined. No direct controls were available for the myoepithelioma strain.

The animals were kept in enamel pans, given water, and fed *ad libitum* with commercial mouse chow, supplemented with hempseed and carrots once weekly.

If the animals were exsanguinated, bleeding was made by heart puncture while they were under nembutal anesthesia; if they were bled repeatedly, the blood was obtained from the jugular vein (8).

*Blood volume determinations.*—Several known procedures were adapted for mice. We began with the exsanguination perfusion (7, 13), followed by the Evans blue (T-1824), I<sup>131</sup>-tagged homologous plasma (4, 14), and P<sup>32</sup>-tagged erythrocyte (9, 10, 14) techniques. All these techniques have their merits and drawbacks. The values differ with the procedure, but by the use of conversion factors they can be integrated (14).

In each experiment the tumor-bearing animals were matched with normals of the same sex, stock, age, and approximate weight. As a rule, only values obtained with the same technic were compared, although they may have been obtained at different times.

Simple exsanguination even without transfusion gives use-

\* Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

† Statistical analysis by J. Moshman.

Received for publication March 12, 1951.

ful information. Either the cell volume or the plasma volume may be determined; the one being measured and the other calculated on the basis of an average assumed body hematocrit—which in mice is about  $\times 0.87$  the large vessel hematocrit. This figure was obtained from simultaneous cell and plasma volume measurements made on twenty mice with the  $P^{32}$ -tagged erythrocyte and T-1824 dye techniques (14). But, as the average body hematocrit of different animals is subject to fluctuations, data calculated on the basis of an average hematocrit can be regarded as only approximate.

In the present series, simultaneous direct cell and plasma volume determinations were made on 27 mice, and the mean value of the conversion factor ( $f$ ) from the average body to the large vessel hematocrit ( $H$ ) was 0.89. If the cell volume ( $C$ ) is known, the total blood volume ( $T$ ) is

$$T = \frac{C}{fH}.$$

If the plasma volume ( $P$ ) is known,

$$T = \frac{P}{1 - fH}$$

and

$$P = T - C \text{ or } C = T - P.$$

If the above conversion values are correct for all animals, simultaneous direct cell and plasma volume determinations should yield identical values, that is:

$$\frac{C}{fH} = \frac{P}{1 - fH}.$$

However, the  $f$  values of the present series varied considerably from group to group (Table 1).

TABLE 1  
MEAN VALUES OF CONVERSION FACTOR ( $f$ ) FROM  
AVERAGE BODY TO LARGE VESSEL  
HEMATOCRIT

Mice	No.	Mean $f$
Granulosa tumor strain V	8	0.865
Ovarian tumor XIX	3	0.932
Normal controls	9	0.846
Breast adenocarcinoma	3	1.048
Normal C3H mice	2	0.833
Myoepithelioma	2	0.946
Total and Mean:	27	0.890

The probability that the means could have been a result of random sampling from the same population was about 0.025.

Inasmuch as most means were the result of a small number of determinations, it was not deemed advisable to correct each strain, and accordingly all data are based on the conversion factor of 0.87 used in earlier studies. Further investigation is indicated to study the variability of  $f$  with the pathological state of the animal.

At present, the best procedure for determination of plasma volume is that made with iodinated albumin. After injection the entire mouse can be lowered in a gamma ionization chamber (12) and the introduced activity precisely ascertained. In order to determine possible extravasation of the injected tagged plasma, the activity of the tail can be measured separately in the same chamber in sacrificed animals. Erythrocytes tagged with  $Fe^{59}$  can serve for determinations of cell volume, using the same chamber, because of the high energy gamma emission of this isotope.

## EXPERIMENTAL

### OVARIAN CARCINOMA ACCOMPANIED BY MODERATE HYPERVOLEMIA WITHOUT ESTROGENIZATION (STRAIN XIX)

In the course of the past 4 years a readily transplantable carcinoma (strain XIX) was secured through successive transplantation of a complex ovarian growth induced by x-rays, as indicated in Chart 1. It is associated with hypervolemia which is due to a moderate plasma volume rise (Table 3) and with cavernous congestion of liver, spleen, and adrenals (Figs. 7-10). None of the tumor-bearing female mice had continued estrus or exhibited other evidence of hyperestrogenization.

*Origin and passages.*—The original tumor induced by x-rays in the right ovary of mouse 1336 measured about 1.5 cm. in diameter; the left ovary measured 2 mm. The bulk of the tumor was solid and grey. The part sectioned showed a predominantly cystic tubular adenoma. The vaginal epithelium was in part in estrus and in part mucinified.

In only one of seven mice (No. 2508) into which fragments of this tumor were implanted was a "take" observed. The bulk of this growth, measuring only  $8 \times 8$  mm. 9 months after implantation, was used to make further subpassages. The small part sectioned still showed a complex, predominantly granular structure.

In the second successive passage the complex appearance persisted. One of the four "takes" was in part a granulosa-cell growth (Fig. 1). The main tumor line derived from mouse 2910 was still complex; the parts shown in Figure 2 contained tubular structures, a few granulosa cells, and large areas of lutein cells seemingly derivatives of granulosa cells. Those shown in Figure 3 contained granulosa-cell clumps with intervening tubular structures and stroma cells.

In the third successive passage a carcinoma with adenomatous structure made its appearance. In the subpassage this adenocarcinomatous structure was retained (Fig. 4). One animal of this transfer generation had a mixed granulosa and luteoma tumor which was not carried on. In the next passages solid medullary carcinomatous areas, as shown in Figure 6, appeared mixed with adenocarcinomatous areas as in Figure 5. The solid areas as illustrated in Figure 6 were the only ones seen in sections of tumors from later transfers. Mitotic figures were abundant.

The rate of growth and percentage of successive transfers and morphological changes in the course of passages are indicated in Chart 1.

*Blood volume determinations.*—The results of

blood volume determinations on mice bearing tumor XIX and those on normal mice are shown in Table 2. The significance of the observed differences in normal and tumor-bearing mice are summarized in Table 3.

There are significant differences among the males in tumor-bearing animals, as compared to

in liver, spleen, and adrenals, these will be illustrated. The degree of congestion shown in Figure 7 is rated + to ++, depending on the extent seen in different fields. When the congestion is mild ( $\pm$ ), one or two cavernous vessels are noted, usually in the portal or subcapsular area of the liver. In the spleen, the cavernous congestion oc-

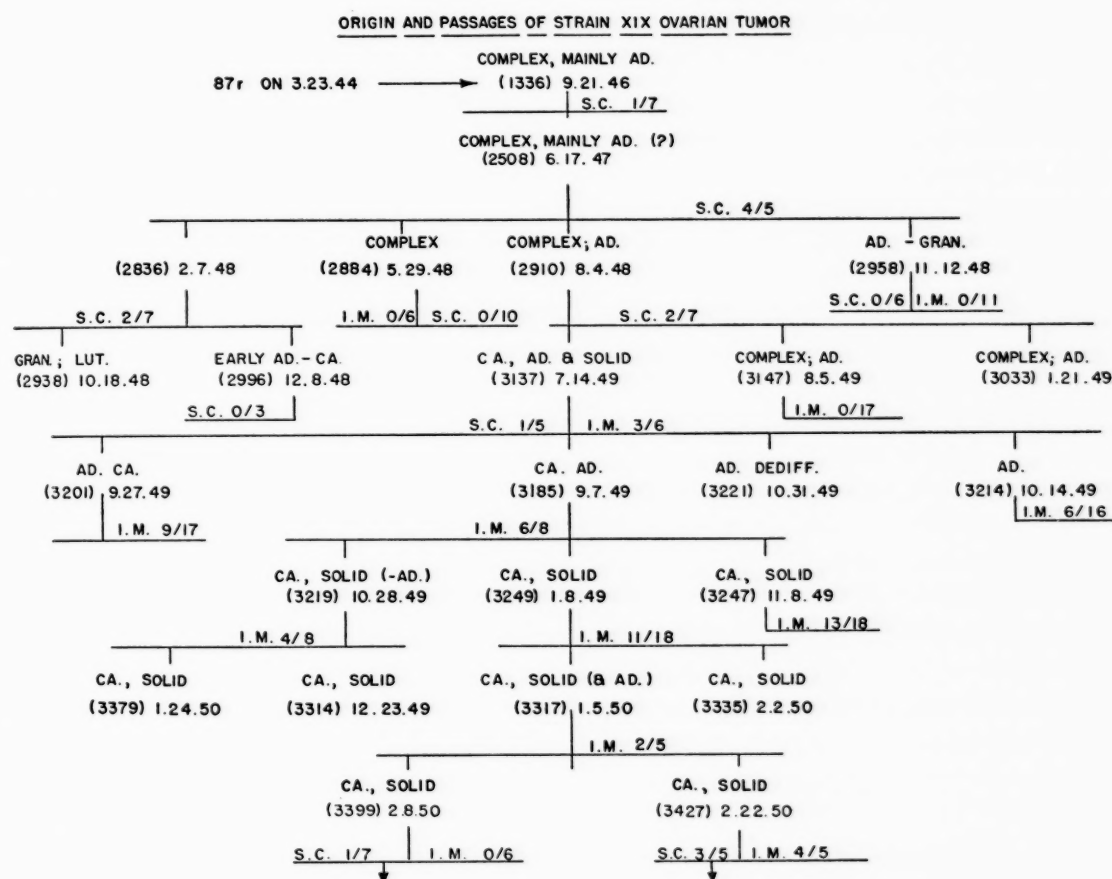


CHART 1.—The fractional figures indicate the number of positives over the number of mice injected. The abbreviations preceding them indicate the route of injection, namely, *s.c.* = subcutaneous, *i.m.* = intramuscular. The figures in parentheses indicate the number of the mouse. The abbrevia-

tions used for the type of tumors are as follows: *ad.* = adenoma, usually tubular in type, *ca.* = carcinoma, *gran.* = granulosa tumor, *lut.* = luteoma, *ca.ad.* = carcinoma, adenomatous in type, *dediff.* = dedifferentiated.

normals for all six indices (Table 3): plasma and cell volumes by direct and indirect determinations, total blood volume and peripheral hematocrits; the plasma and total blood volumes were higher in tumor-bearing males, whereas the cell volumes and hematocrits were lower than in normal controls. In tumor-bearing females only the drop in hematocrits is significant, but the changes in all indices are in the same direction as in males.

**Congestive changes.**—Since this is the only tumor strain not of the granulosa-cell type in which we found congestive changes of the hypervolemic type

occurs in the pulp (Fig. 8). The follicles appear isolated by a surrounding pool of blood in cavernous sinuses. The degree of congestion in Figure 8 is rated ++ to +++. In the adrenal the congestion is invariably in the cortex, and the medulla is spared (Figs. 9, 10). The degree of congestion in Figure 9 is rated +++, that in Figure 10 is rated ++. The preference seems to be for the mid-part of the cortex, and when the congestion is advanced the cortical cells undergo pressure atrophy and drop out. The blood in cavernous vessels is frequently leukemoid (Fig. 9); leukocyte

precursors are few. The congestive changes occurred in both sexes, although they seem more conspicuous in males.

#### TRANSPLANTABLE MAMMARY TUMORS

##### BREAST CARCINOMA M WITH SLIGHT HYPERVOLEMIA AND MARKED OVARIAN CONGESTION

This transplantable breast tumor carried in C3H mice was characteristically associated with a cavernous congestion of the ovary (Fig. 12) with no, or only very slight, congestion of other organs. In sections the tumor appears to be a common type of mammary adenocarcinoma. Grossly, the ovaries of mice bearing large tumors of this strain

were greatly swollen and red, suggesting the presence of acute hematomas. But the sections showed that the blood was contained entirely within cavernous spaces lined by endothelium, as noted frequently in granulosa tumor-bearing mice. The maximum degree of congestion encountered in other organs is illustrated in Figure 11 (liver) and Figure 13 (adrenal). The congestion in Figure 11 is rated  $\pm$  or  $+$  but cannot be regarded as distinctly cavernous and focal. Congestion in Figure 13 (adrenal), however, is of the cavernous type but only of a degree rated as  $\pm$ .

Blood volume determinations made on twelve mice (Table 2) indicate a significantly elevated

TABLE 2  
SUMMARY BLOOD AND TUMOR VOLUME DETERMINATIONS IN MICE

MOUSE STRAIN	No. OF MICE*	TUMOR SIZE†	VOLUME OF				TOTAL BLOOD VOLUME	PERIPHERAL HEMATOCRIT‡ (per cent)
			Direct	Cells Indirect (ml.)	Plasma Direct (ml.)	Indirect		
Ovarian ca. XIX (7 females)	7	Max. ++++	1.16	2.77	9.60	7.52	12.37	33.4
		Min. +++±	1.16	1.01	3.97	7.52	5.60	17.2
		Mean ++++	1.16	1.69	5.75	7.52	7.61	25.74
		S.E.		0.24	0.81		0.87	2.1
Ovarian ca. XIX (10 males)	10	Max. ++++	2.70	2.75	10.60	9.90	12.58	34.9
		Min. +++±	1.80	1.98	6.30	6.61	8.53	18.0
		Mean +++±	2.23	2.36	7.90	7.91	10.20	25.85
		S.E.	0.17	0.16	0.93	0.43	0.43	1.7
Normal Rf/Ak (12 females)	12	Max.	4.00	4.46	6.50	6.23	10.23	51.51
		Min.	3.30	1.50	3.00	4.90	4.50	33.71
		Mean	3.65	2.92	4.99	5.49	8.32	42.98
		S.E.	0.10	0.71	0.33	0.22	0.47	1.50
Normal Rf/Ak (25 males)	25	Max.	3.90	3.75	5.56	6.06	9.84	50.41
		Min.	3.10	1.90	4.00	4.89	5.80	35.61
		Mean	3.48	2.84	4.83	5.42	8.01	42.84
		S.E.	0.08	0.11	0.11	0.12	0.12	0.67
Breast ca. R (10 females, 4 males)	14	Max. ++++		3.80	10.35		14.15	30.61
		Min. +		0.69	5.40		6.67	8.11
		Mean +++		1.94	7.58		9.49	22.50
		S.E.		0.30	1.05		0.89	2.22
Myoepithelioma (5 females)	5	Max. +++	3.56	3.22	6.60	7.03	9.98	38.71
		Min. ++	3.48	2.36	5.11	7.00	7.47	36.41
		Mean +++±	3.52	2.86	5.89	7.02	8.91	37.38
		S.E.	0.04	0.17	0.30	0.01	0.51	0.46
Granulosa tumor V (22 females)	22	Max. ++++	5.68	5.20	23.30	14.10	25.20	46.11
		Min. +	3.00	1.90	6.18	6.34	10.05	8.91
		Mean +++±	3.78	3.29	10.63	11.09	14.23	26.20
		S.E.	0.35	0.20	0.90	1.14	0.74	2.42
Granulosa tumor V (19 males)	19	Max. ++++	4.44	4.67	15.84	11.34	19.18	47.21
		Min. +	3.20	1.75	3.98	5.28	5.79	18.51
		Mean +++	3.62	3.00	9.09	9.15	11.90	30.43
		S.E.	0.17	0.20	0.77	0.97	0.81	1.94
Breast ca. M (12 females)	12	Max. ++++	2.80	2.70	18.50	20.70	23.20	19.11
		Min. ++	2.00	0.40	3.30	14.20	3.70	9.41
		Mean +++±	2.43	1.59	11.19	17.07	13.39	13.75
		S.E.	0.52	0.20	1.24	1.92	1.58	1.02
Normal C3H (6 females, 1 male)	7	Max.	2.6	2.8	5.6	5.1	8.00	39.11
		Min.	2.3	1.5	2.7	5.1	4.20	34.91
		Mean	2.45	2.29	4.64	5.1	6.57	36.51
		S.E.	0.14	0.14	0.70	0.00	0.48	0.64

\* In several cases further readings were made at irregular periods after the first test. These repeated observations were counted neither in the number of different mice nor in the tabulation in the body of the table.

† Tumor sizes of less than 1 cm. in the average diameter are indicated as  $\pm$ , those between 1 and 2 cm. by +, between 2 and 3 cm. by ++, between 3 and 4 cm. by +++, and 4 cm. and over by ++++.

‡ These were made by the capillary technic of Parpart (cf. 8).

plasma and total blood volume and a slightly to moderately reduced cell volume, the latter being significant only in the groups in which indirect determinations were made. The hematocrits are very low. So, the elevation of plasma volume is much in excess of that needed to compensate for loss of cell volume.

The lack of characteristic congestive changes in liver, spleen, and adrenals in the presence of markedly elevated plasma and total blood volume remains to be explained. A marked congestion of the ovary, characteristic of this strain, was advanced in three mice with moderately elevated blood volume and in one with normal blood volume. It also remains to be determined whether a specific relation exists between mammary gland and ovarian congestion. An isolated cavernous congestion of the ovary in an animal with normal blood volume can only be explained by a vasodilator substance with specific affinity for the ovary.

#### TRANSPLANTED BREAST TUMOR WITH SLIGHT HYPERVOLEMIA AND NO CONGESTIVE CHANGES (STRAIN R)

Blood volume determinations made on tumor-bearing mice of this strain are summarized in Table 2. In this series, the total blood volume was

slightly and the plasma volume moderately elevated. The hematocrit values were moderately below normal and cell volumes slightly so. All but the latter changes are statistically significant.

#### SPONTANEOUS BREAST TUMORS

Numerous mice with very large spontaneous breast tumors were studied earlier by the dye technic (7, 13). The current series includes two C3H mice with large breast tumors whose blood volume was assayed also by the dye technic. The following values were obtained:

Total blood volume, 11.76 and 13.0 per cent  
Plasma volume, 8.3 and 10.2 per cent  
Cell volume, 3.46 and 2.8 per cent  
Hematocrit values, 33.8 and 24.8 per cent

Thus, in conformity with earlier findings, these mice with spontaneous breast tumors had a slightly elevated plasma and total blood volume, but none had the cavernous congestive changes characteristic of granulosa tumor-bearing mice.

#### AN X-RAY-INDUCED OVARIAN CARCINOMA WITH ADVANCED LIVER CONGESTION

Most, if not all, ovarian tumors induced by x-rays are localized to the sites of origin, even

TABLE 3  
SIGNIFICANCE OF DIFFERENCES BETWEEN TUMOR-BEARING AND CONTROL MICE BY SEX

MICE	CELL VOLUME		PLASMA VOLUME		TOTAL BLOOD VOLUME (ml.)	PERIPHERAL HEMATOCRIT (per cent)
	Direct	Indirect (ml.)	Direct	Indirect (ml.)		
Male:						
Ov. ca. XIX	2.23	2.36	7.90	7.91	10.20	25.85
Normal controls	3.48	2.84	4.83	5.42	8.01	42.84
Difference	-1.25	-0.48	3.07	2.49	2.19	-16.99
Probability	<0.01	0.02	<0.01	<0.01	<0.01	<0.01
Female:						
Ov. ca. XIX	1.16	1.69	5.75	7.52	7.61	25.74
Normal controls	3.65	2.92	4.99	5.49	8.32	42.98
Difference	-2.49	-1.23	0.76	2.03	-0.71	-17.24
Probability	*	0.12	0.42	*	0.48	<0.01
Male and female:						
Breast ca. M	2.43	1.59	11.19	17.07	13.39	13.75
Normal controls	2.45	2.29	4.64	5.10	6.57	36.51
Difference	-0.02	-0.70	6.55	11.97	6.82	-22.76
Probability	0.97	<0.01	<0.01	<0.01	<0.01	<0.01
Male and female:						
Breast ca. R		1.94	7.58		9.49	22.50
Normal controls		2.29	4.64		6.57	36.51
Difference		-0.35	2.94		2.92	-14.02
Probability		0.31	0.04		<0.01	<0.01
Male:						
Granulosa tumor V	3.62	3.00	9.09	9.15	11.90	30.43
Normal controls	3.48	2.84	4.83	5.42	8.01	42.84
Difference	0.14	0.16	4.26	3.73	3.89	-12.41
Probability	0.46	0.48	<0.01	<0.01	<0.01	<0.01
Female:						
Granulosa tumor V	3.78	3.29	10.63	11.09	14.23	26.20
Normal controls	3.65	2.92	4.99	5.49	8.32	42.98
Difference	0.13	0.37	5.64	5.60	5.91	-16.78
Probability	0.73	0.20	<0.01	<0.01	<0.01	<0.01

\* Probability unassessable due to reading in a single female.

though they may appear malignant in sections and frequently replace the entire ovary. The congestive changes in relation to spontaneous or x-ray-induced ovarian tumors have thus far not been systematically studied; but, presumably, they are rare, as they do not occur with transplanted tumors until the latter are large. The spontaneous ovarian tumors seldom exceed 2 cm. in diameter.

This mouse (BR 264) received 300 r at 5 months of age and died at 20 months. In sections, the bulk of the tumor appeared necrotic or replaced by fat-laden cells, empty cavities, and sinusoids and clefts apparently at sites of dissolved cholesterol crystals (Fig. 14). The few "healthy" cells present were of the granulosa type, and in the metastases they distended arterioles and small arteries (Fig. 15). The advanced congestion of the liver was accompanied by a mild leukemoid reaction (Fig. 16).

#### MISCELLANEOUS NEOPLASMS WITH NO CONSPICUOUS BLOOD VOLUME RISE OR CONGESTIVE CHANGES

Sections from five mice bearing large transplanted sarcomas gave no indications of a hypervolemic congestion.

Blood volume determinations on five mice with transplanted myoepitheliomas are shown in Table 2. All mice bearing the myoepithelioma were females and had no direct controls. To gain a rough idea of their position in the over-all picture, the various means of blood indices of the different females were all ranked in ascending order of magnitude in Table 4. It may be observed that

TABLE 4

MEAN RANKINGS OF FEMALE MICE WITH RESPECT TO VARIOUS BLOOD INDICES

INDEX	MYOEPI- TH.	GRANU- LOSA			
		OV. CA. XIX	BREAST CA. R	STRAIN V	NORMAL R/A CSH
Direct cell volume	3	1		5	4 2
Indirect cell volume	4	1.5	1.5	6	5 3
Direct plasma volume	4	3	5	6	2 1
Indirect plasma volume	3	4		5	2 1
Total blood volume	4	2	5	6	3 1
Peripheral hematocrit	5	2	1	3	6 4

mice bearing myoepithelioma were intermediate to both controls with respect to direct and indirect cell determinations and the hematocrit. Their plasma and total blood volumes were higher. The numbers involved are too small to make any exact tests of significance, but it is noteworthy that the hematocrit mean of 37.38 greatly exceeds that of the other tumor strains, but is within the normal range.

#### COMPARATIVE OBSERVATIONS WITH GRANULOSA TUMOR, STRAIN V

Simultaneous comparative determinations with a known granulosa tumor seemed necessary to

evaluate the blood volume values in mice with miscellaneous other tumors. In the course of 5 years of consecutive passages this strain has changed considerably. The original strain isolated in 1944 (1) was extensively studied in 1948 (8), but it still possesses some of the salient features of a granulosa tumor. Originally a typical granulosa-cell growth (Fig. 17), it is now an anaplastic carcinoma (Fig. 18), appearing in some sections as a syncytial-like growth (Fig. 19). The latter two forms are noted in the same tumor, and it is not clear to us whether this difference is due to processing or to the state of cells in the growths. Occasional metastases are seen, as in the original tumor, in liver and lung (Fig. 21) in the form of tumor emboli.

The characteristic focal and cavernous congestive changes of the original tumor have been retained (Fig. 20) but not their degree. That in Figure 20, rated as ++, is most frequently encountered at autopsy, but higher and lower grades of congestion do occur with large tumors.

Blood volume determinations made in the course of these studies are summarized in Table 2.

There is a significant rise in plasma and total blood volume by both direct and indirect determinations. The red cell mass, in contrast to that of hosts bearing other types of tumors, was actually above normal, although the rise is not statistically significant. Nevertheless, the hematocrits dropped significantly below normal. Thus, it is evident that with granulosa tumors the plasma volume rise could not have been secondary to anemia.

#### INFLUENCE OF SEX OF TUMOR-BEARING HOSTS ON BLOOD VOLUME

Since androgens are known to raise the cell volume (8) and all granulosa tumors studied were associated with a plasma volume rise, it seemed desirable to analyze the relation of sex to blood volume as shown in Table 5.

Three measures apparently give results significant on the 0.05 level, but it should be borne in mind that the concept of a 5 per cent level of probability implies that errors are expected that proportion of the time. In a long series of successive significance tests, this random process will yield false significant results by chance a number of times. Three out of twenty cases are not enough categorically to conclude these to be true differences.

The greater cell volume in male mice bearing tumors XIX recalls that of mice with masculinizing luteoma (8). The greater plasma volume rise in female mice is noteworthy, since congestive changes and hypervolemia occur most regularly in

mice bearing female sex hormone-secreting neoplasms.

### DISCUSSION

Cell, plasma, and total blood volume determinations were made in the course of current studies on diverse tumors to determine whether hypervolemia and cavernous congestion of viscera are specific to granulosa tumors. Analysis of the data indicates that there is a statistically significant rise of plasma and total blood volume in male and female mice bearing a transplanted granulosa and two strains of breast tumors (M and R) and in

and carcinoma XIX—all of ovarian origin. With large granulosa tumors cavernous congestion is common and marked, while it is absent or inconspicuous with the breast tumors studied by us.

Recently, Dickie and Woolley (3) have observed a transplanted neoplasm, presumably of mammary origin, in which the congestive changes were similar to those seen with granulosa tumors. Furthermore, Wolstenholme and Gardner (15) described a testicular neoplasm of interstitial-cell origin with cavernous dilatation of sinusoids in liver, spleen, and adrenals. This testicular tumor,

TABLE 5  
SIGNIFICANCE OF SEX DIFFERENCES ON BLOOD VOLUME VALUES WITHIN  
VARIOUS STRAINS OF MICE

MICE	CELL VOLUME		PLASMA VOLUME		TOTAL BLOOD VOLUME (ml.)	PERIPHERAL HEMATOCRIT (per cent)
	Direct	Indirect (ml.)	Direct	Indirect (ml.)		
Ovarian ca. XIX:						
Male	2.23	2.36	7.90	7.91	10.20	25.85
Female	1.16	1.69	5.75	7.52	7.61	25.74
Difference	1.07	0.67	2.15	0.39	2.59	0.11
Probability	*	0.05	0.10	*	0.02	0.97
Breast ca. R:						
Male		2.49	7.41		9.91	27.02
Female		1.69	7.65		9.30	20.50
Difference		0.80	-0.24		0.61	6.52
Probability		0.19	0.88		0.72	0.06
Granulosa tumor strain V:						
Male	3.62	3.00	9.09	9.15	11.90	30.43
Female	3.78	3.29	10.63	11.09	14.23	26.20
Difference	-0.16	-0.29	-1.54	-1.94	-2.33	4.23
Probability	0.68	0.26	0.20	0.22	0.03	0.17
Normal Rf/Ak:						
Male	3.48	2.84	4.83	5.42	8.01	42.84
Female	3.65	2.92	4.99	5.49	8.32	42.98
Difference	-0.17	-0.08	-0.16	-0.07	-0.31	-0.14
Probability	0.22	0.91	0.67	0.78	0.55	0.94

\* Probability unassessable due to single female reading.

males bearing a nonhormone-secreting ovarian carcinoma (XIX).<sup>1</sup>

The peripheral hematocrits drop significantly in all tumor-bearing hosts, but there is a difference between granulosa and other tumors. The red cell mass is below normal in the latter but not in the former. It is possible that a drop in red cell volume is compensated by a rise in plasma volume, but this cannot be true for granulosa tumors in which the plasma volume rise is independent. Furthermore, there is a discrepancy between blood volume values and congestive changes: in the breast tumor strains studied the plasma volume was about as high as that of strains V and XIV (granulosa) and XIX (ovarian carcinoma), but the characteristic congestive changes in liver, spleen, and adrenals were noted only with granulosa tumors V and XIV.

<sup>1</sup> A preliminary survey was presented at the Fifth International Cancer Congress (10). The present report amplifies and corrects the preliminary tabulation.

having no hormonal activity, may be a counterpart of our ovarian tumor XIX. Similarly, in mice bearing luteomas (8) there is a slight hypervolemia, owing primarily to an elevated cell volume, with very occasional cavernous congestion of viscera.

Mammary gland tumors are not known to secrete hormones, but their origin is related to estrogens. Thus, all tumors accompanied by hypervolemia now known have some connection with sex hormone-secreting cells. Data on blood volume changes in mice with other related steroid hormone-secreting neoplasms, e.g., those originating in the adrenal cortex or in Sertoli cells, are not available. Such data supplemented by infrared assays of steroids and their metabolites in tumor-bearing hosts may identify the hypothetical substance (plethorin) assumed to be directly responsible for the blood volume rise.

More data are needed on the presence or ab-

sence of congestive changes and hypervolemia in mice bearing large transplanted tumors not related to sex hormones. The observations made thus far suggest that active estrogens alone are not the cause of the hypervolemia.

Indirect evidence was sought for the quantity of estrogen secreted by granulosa tumors by the *in vivo* neutralization test (6). When the granulosa tumors studied reached a size of approximately 2 cm. in diameter, the spayed females went into a continued estrus, as indicated by vaginal smears. To such tumor-bearing mice, testosterone was given daily in quantities up to 5 mg., which is sufficient to neutralize about 250 times the estrus maintenance dose of estradiol. Estrus was abolished in only one of eight mice of this series, indicating that a tremendous amount of hormone is discharged by the tumor, but even in this mouse marked congestive changes were present in characteristic locations. Yet, the plasma volume of five of these mice measured was only moderately elevated. It ranged from 7.8 to 11.4 per cent of body weight, with a mean of 9.2 per cent. The hematocrit of these mice ranged from 24.9 to 39.5, with a mean of 33.9 per cent.

In studying the relationship of tumor size and the various hematological indices it was observed that, after separating the readings into dichotomous classes of those with tumor size 3 (between 3 and 4 cm. in diameter) or higher and those of less than 3, the data could not very well fulfill assumptions or normality. Accordingly, differences were tested by a nonparametric test of Pitman (11), which involves no such assumption.

Of all the indices (or measurements) considered, only the hematocrit in the strain V granulosa tumor group differed at the 0.05 level. Mice with the smaller sized tumors had a mean hematocrit of 33.4, compared to 27.9 for the mice with larger tumors. The changes with other tumors were in the same direction. In this study only large tumors were analyzed. In earlier studies, the onset and progressive growth of neoplasms were correlated with blood volume changes (1, 7, 8, 13), and it was found that at first there is a direct relationship between tumor size and blood volume rise, but after the tumor reaches a certain size (about ++++) there may be no further rise in blood volume, and even a drop may occur.

Organ blood volume determinations to be reported more fully have disclosed an excessive amount of blood in certain viscera as was expected, but, unexpectedly, very low blood volumes in the tumors. In the latter the plasma content was 0.01–0.04 cc. per gram of tumor, as compared to 0.12–0.16 in normal and 0.25–0.30 cc. in hyper-

volemic livers. The cell volumes were similarly low in tumors. These findings suggest that it is not an increased blood mass in the tumor which causes an over-all rise of blood volume. The possible causes of pathogenesis of hypervolemia have already been discussed (13). As a working hypothesis, we assumed that hypervolemia is due to a disturbance of the physiological mechanism which maintains the blood volume and that this operates through albumin production (7, 13), mediated by a special substance tentatively named "plethorin" which is present in excess in mice bearing certain tumors.

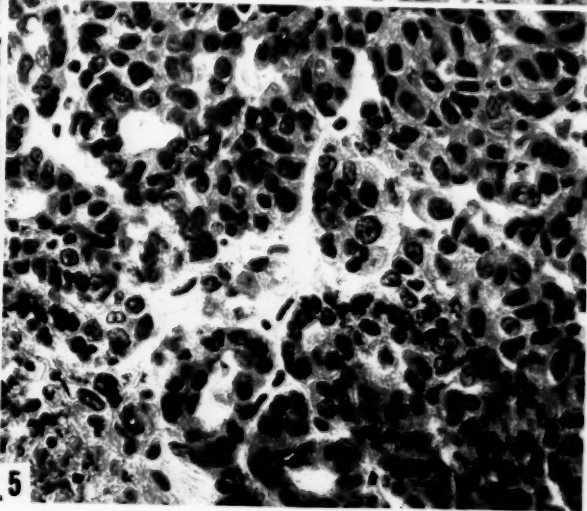
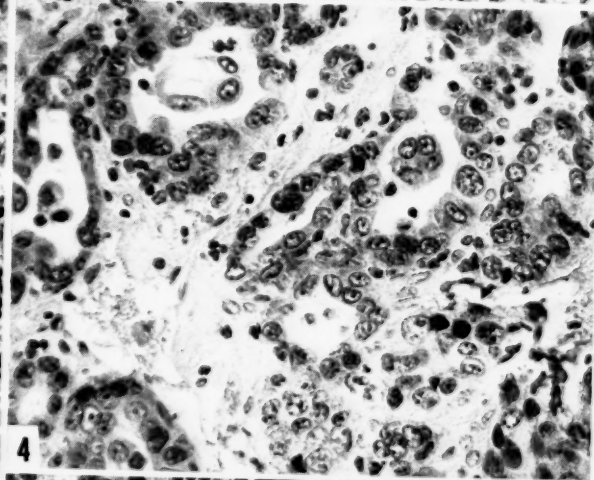
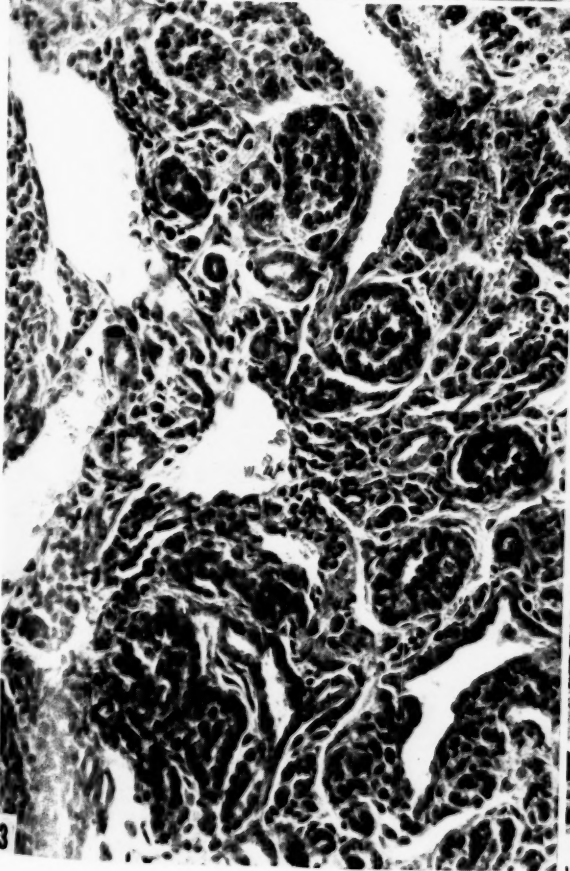
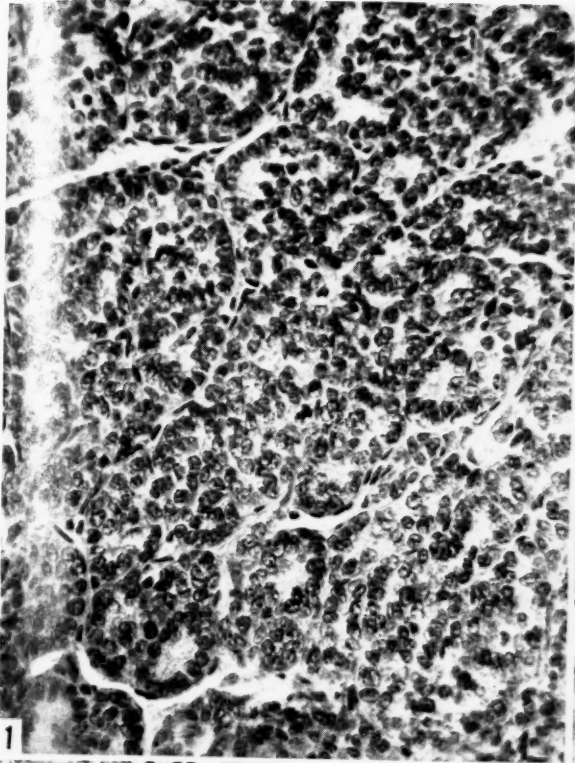
Future research aimed to elucidate the phenomena here described should include: (a) further blood volume measurements coupled with morphological studies of congestive changes in animals bearing neoplasms unrelated to sex hormones, (b) assays of steroids and their metabolites in tumor-bearing hosts, (c) search for an assay method of the hypothetical plethorin, (d) study procedures and substances which might raise the blood volume of normal mice or influence that of granulosa tumor-bearing mice, and (e) attempts to isolate plethorin by chemical and physical methods.

#### SUMMARY AND CONCLUSIONS

This is a comparative study of plasma and cell volumes and congestive changes in estrogen-secreting granulosa tumors and several non-hormone-secreting neoplasms. A significant increase in blood volume was found in mice bearing transplantable (a) ovarian carcinomas (strain XIX), (b) breast tumors (strains M and R), and (c) granulosa tumors (strain V).

Cavernous congestion of many viscera was frequent and marked with granulosa-cell tumors, even in the absence of extreme elevation of plasma volume. Such congestion was absent in mice with breast tumors, strain R; it was localized to the ovaries in breast tumor strain M, and was slight but widespread with the ovarian carcinoma XIX. This ovarian carcinoma strain was derived from a complex x-ray-induced ovarian tumor and histogenetically appears related to granulosa tumors. The successive transformation of this strain into an anaplastic carcinoma is described.

Common to all strains of tumors accompanied by hypervolemia was a drop in hematocrits and rise in plasma volumes. There was, however, this difference between the granulosa and the other tumors causing hypervolemia: with all but the ovarian strains, the erythrocyte volumes were below normal. Thus, the plasma volume rise with granulosa tumors is not secondary or compensatory to a drop in erythrocyte mass, as might be the case with other tumors.



All sections were stained with hematoxylin and eosin. The magnifications are approximate.

FIG. 1.—Ovarian tumor of strain XIX, second successive passage (mouse No. 2958), showing a characteristic granulosa pattern.  $\times 230$ .

FIG. 2.—Ovarian tumor of strain XIX, second successive passage (mouse No. 2910), showing mixed composition with tubular structures, lutein-like cell masses, granulosa and stroma cells.  $\times 230$ .

FIG. 3.—Ovarian tumor of strain XIX. Another area of same tumor (2910) showing better defined granulosa follicles and stroma cells.  $\times 400$ .

FIG. 4.—Ovarian tumor of strain XIX, fourth successive passage (mouse No. 3185), showing a well differentiated adenocarcinoma.  $\times 400$ .

FIG. 5.—Ovarian tumor of strain XIX, sixth successive passage (mouse No. 3317), showing carcinoma with solid and glandular areas.  $\times 400$ .

FIG. 6.—A higher magnification of the solid area of the same tumor (3317), showing syncytium-like masses with many mitotic figures.  $\times 950$ .

FIG. 7.—Moderate cavernous congestion of liver sinusoids of a mouse (3309) bearing ovarian carcinoma, strain XIX.  $\times 100$ .

FIG. 8.—Characteristic moderate hypervolemic type of congestion of the spleen in a mouse (3497) bearing ovarian carcinoma, strain XIX.  $\times 100$ .

FIG. 9.—Advanced congestion of the adrenal of a mouse (3335) bearing a transplanted ovarian tumor XIX. Most of the blood is in the middle part of the cortex. Some of the intact medulla is seen in the right lower corner of the field.  $\times 230$ .

FIG. 10.—Moderate congestion of the adrenal cortex of mouse 3497 bearing tumor strain XIX. The intact medulla is in the right upper corner of the field.  $\times 230$ .

FIG. 11.—Slight diffuse congestion of liver not with certainty of the hypervolemic type. This was the maximum degree of congestion encountered in the liver of mice bearing carcinoma strain M (HO 207).  $\times 100$ .

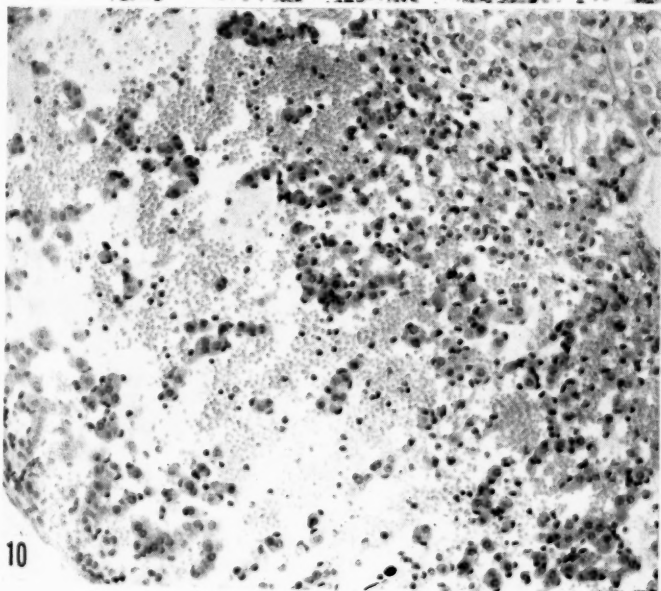
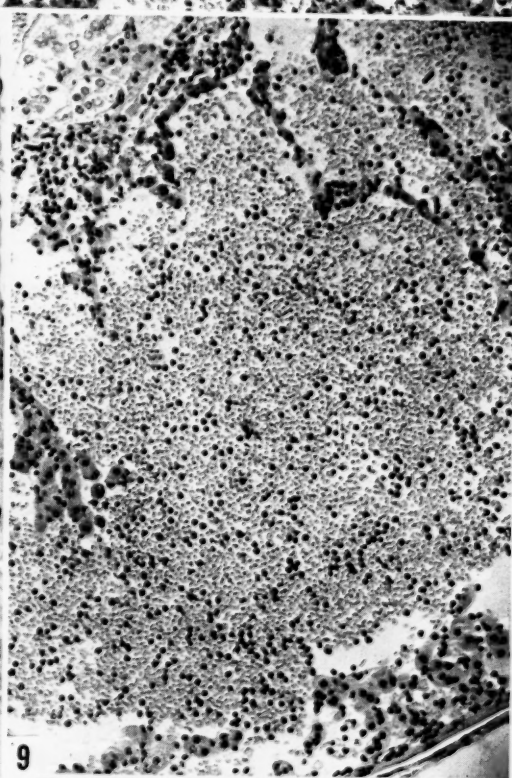
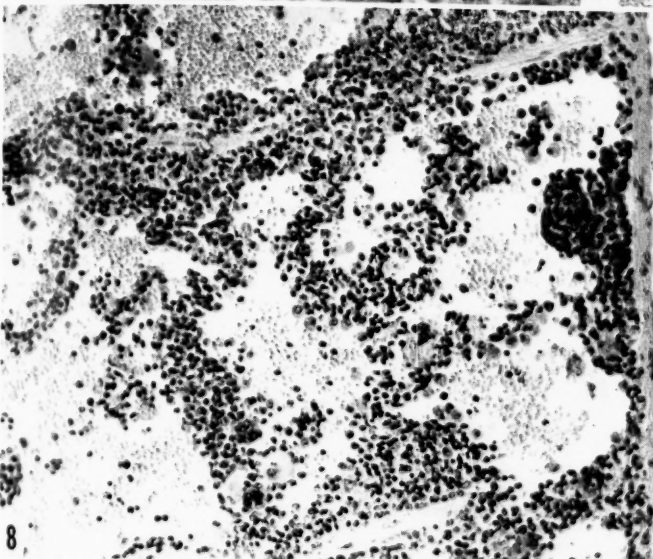
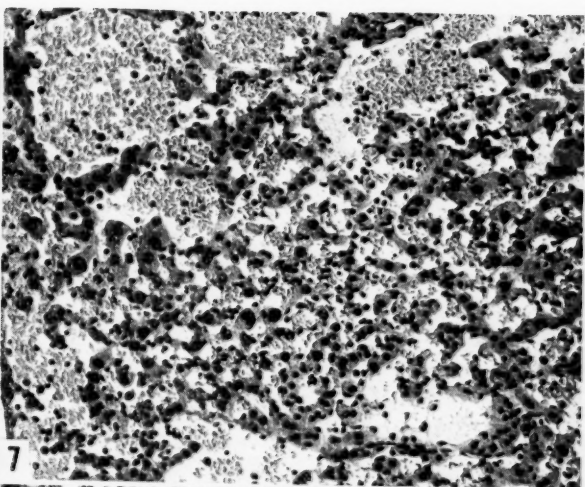
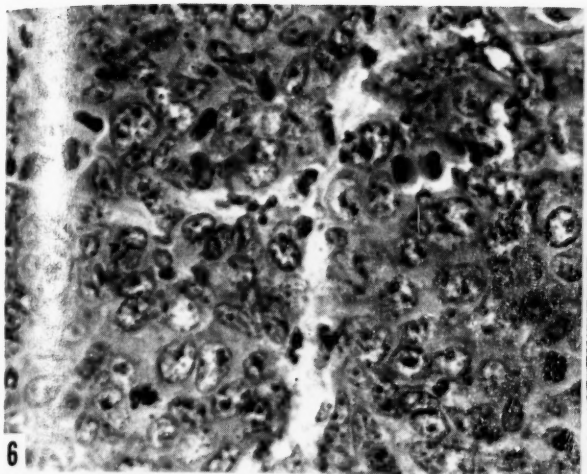


FIG. 12.—Advanced cavernous congestion of the ovary of a mouse (HO 207) bearing transplanted adenocarcinoma, strain M.  $\times 100$ .

FIG. 13.—Incipient cavernous congestion in subcapsullary area of the adrenal cortex of a mouse (HO 204) bearing transplanted breast carcinoma, strain M.  $\times 100$ .

FIG. 14.—Representative microscopic field of the x-ray-induced ovarian tumor BR 264. The bulk of the growth is composed of necrotic tissue with foam cells, debris, and clear spaces; some of the latter are shaped as cholesterol crystals. There are clumps of granulosa cells and a vascular granulation tissue.  $\times 100$ .

FIG. 15.—Two small arteries of the lung distended with granulosa cells. Mouse BR 264 bearing an x-ray-induced ovarian carcinoma.  $\times 100$ .

FIG. 16.—Moderate congestion of the liver of the same animal.  $\times 100$ .

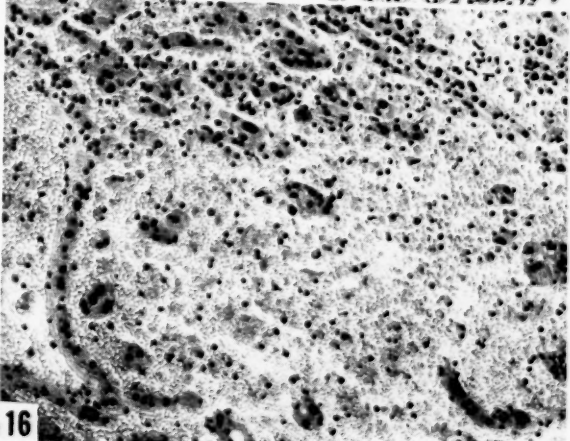
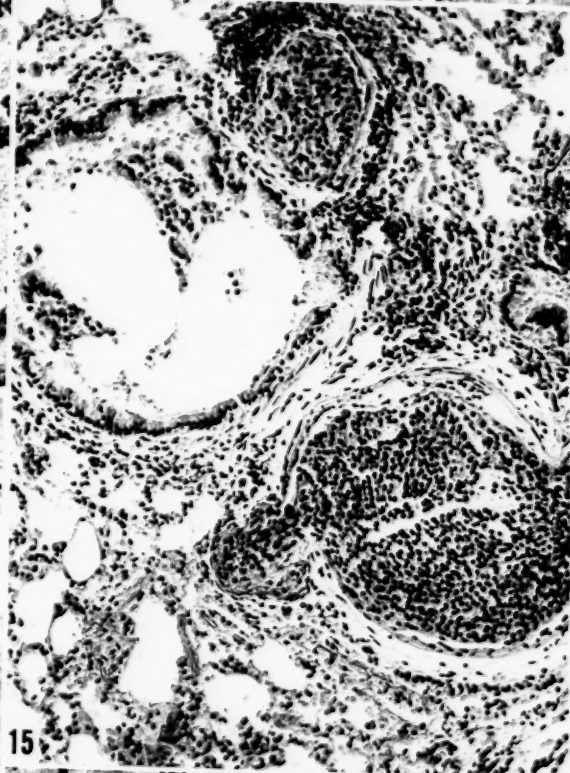
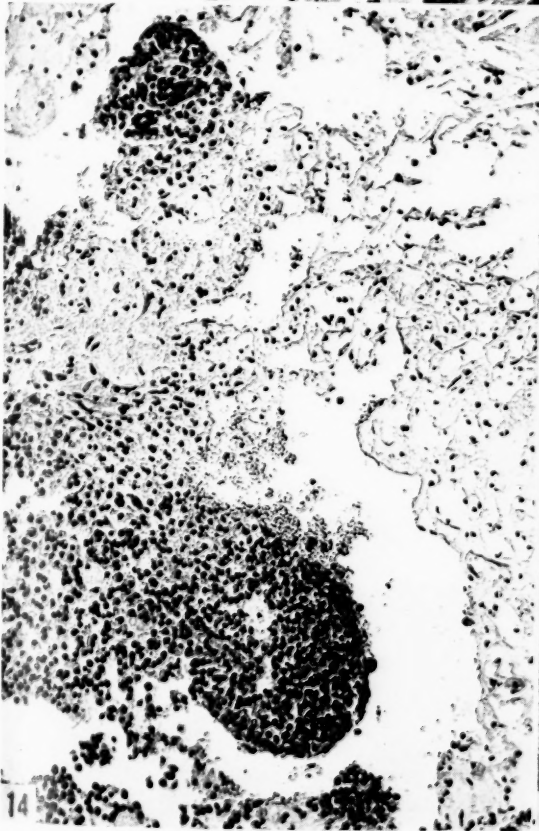
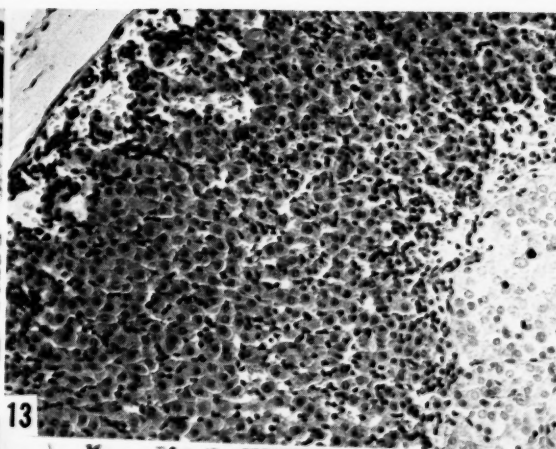
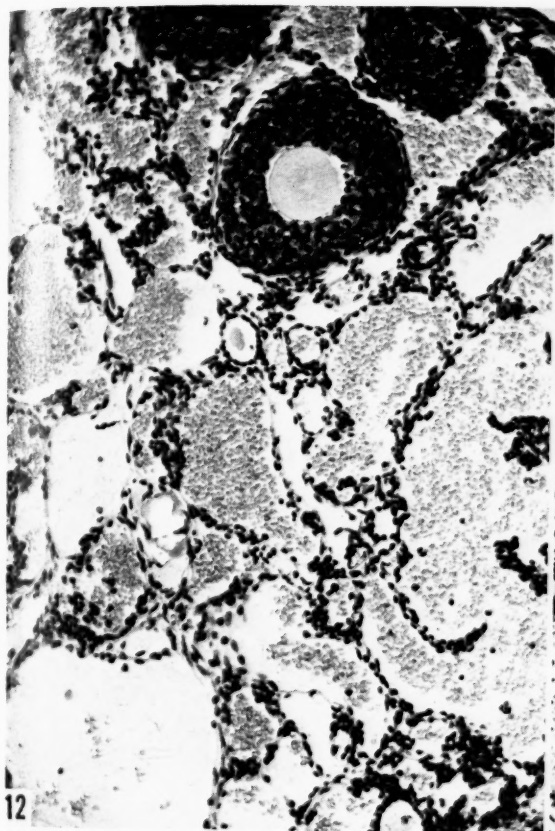
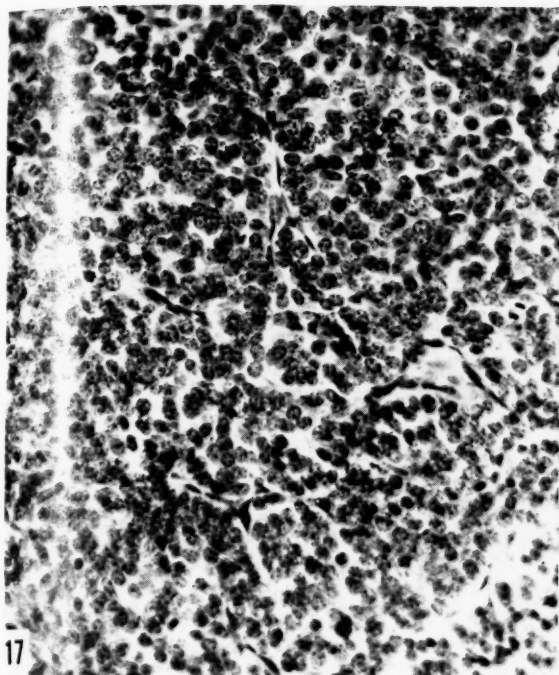


FIG. 17.—Microscopic appearance of the granulosa tumor, strain V, in 1944.  $\times 230$ .

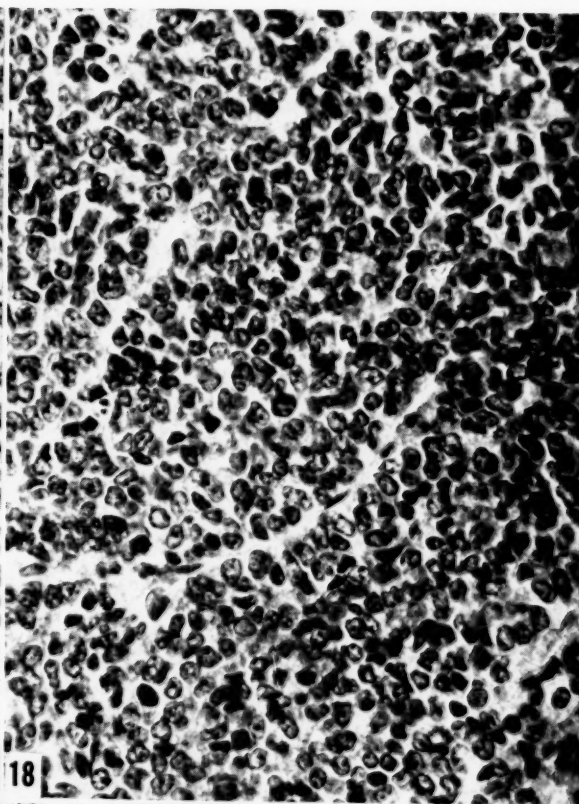
FIGS. 18, 19.—Characteristic microscopic appearance of the granulosa tumor, strain V, 1950.  $\times 400$ .

FIG. 20.—Characteristic moderate hypervolemic type of congestion of the liver of a mouse (3517) bearing granulosa tumor of strain V.  $\times 230$ .

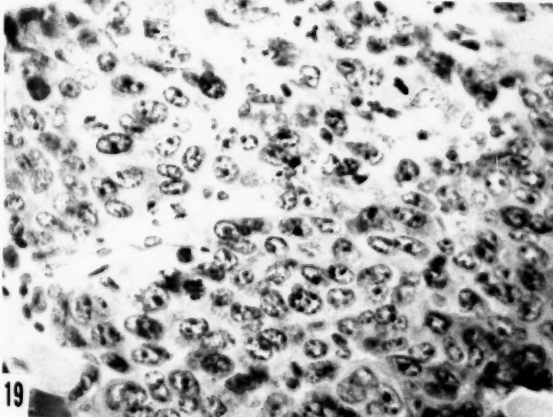
FIG. 21.—Tumor embolus in the lung of a mouse (3704) bearing granulosa tumor, strain V.



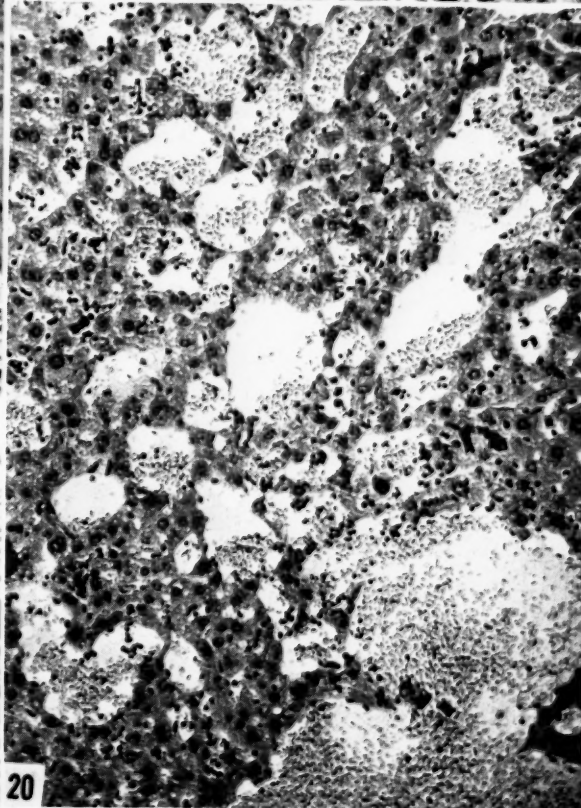
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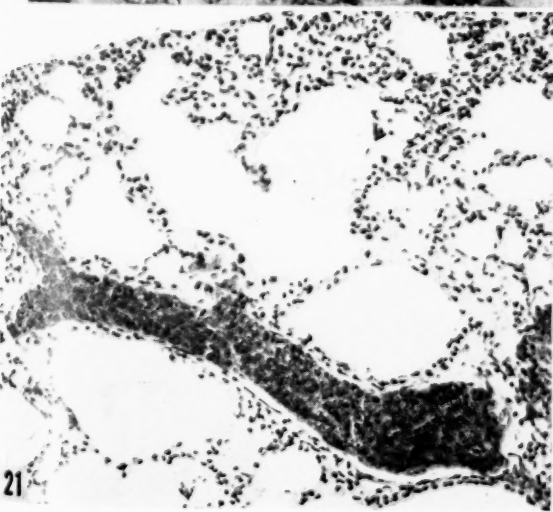
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Attention is called to a remarkable relationship between a transplantable breast tumor and secondary hemangioma-like congestion of the ovary.

Earlier observations on the relative oligemia of tumors are confirmed.

#### ACKNOWLEDGMENTS

A few values cited were obtained in experiments with Mr. R. H. Storey and Dr. J. B. Kahn. Misses M. M. Knoohuizen and E. J. Beale assisted in the work. Mr. W. D. Gude made the sections and the photomicrographs. Mr. L. Wish helped in matters of radioisotope technics; this was possible through the sustained interest of Dr. C. W. Sheppard.

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# A Method for Determining Mutation Rate and Tumor Incidence Simultaneously\*

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Interest in the possible relationship between somatic mutations and the etiology of cancer has focused attention on chemicals which have mutagenic or carcinogenic properties or both. Evidence obtained from experiments with mice has been gained by recording mutation rates of visible characters after treatment with compounds known to produce tumors in that species (6). Mutation rates have also been determined after treating *Drosophila* with chemicals by various methods (1, 3, 4) and then observing the number of lethals on the X chromosome. Bacteria and fungi may also be used for inducing mutations (2, 5), but tumors do not appear in these organisms. A desirable and critical test should consist of simultaneous determination of both lethal mutation rate and tumor incidence following treatment. This can be done in *Drosophila*, and an arrangement of matings in tumor stocks of *Drosophila* has been devised by which it is possible to determine tumor incidence and mutation rate at the same time.

The advantages of this method are that mutation rate and tumor incidence are tested in the same group of animals, treatment is identical for determinations of mutation rate and tumor incidence, the same control group of flies is utilized for both determinations, the number of experiments where negative results are obtained is decreased, since the chemical may affect tumor incidence even if it does not change mutation rate (and vice versa), mutation rate is measured objectively, and large numbers of individuals may be tested in a short time. The validity of the results rests on the identity of pigmentation with previous cellular tumors (3) and the similarity of these growths in *Drosophila* to tumors in mammals.

An outline of the appropriate crosses is presented in Chart 1. It has been found that, although theoretically it is preferable to use individual matings, it is more feasible from a prac-

tical standpoint to use multiple matings in bottles. To eliminate the possibility that lethals may be carried by the parental stocks before treatment, individual matings are made the generation before the experiment is begun, and counts of the sex ratio of siblings of flies used in the experiment are made. No individual is used when such a ratio is aberrant. The entire experiment should be done at a constant temperature, preferably in an incubator. A constant amount of formula should be used for the food and vials, and bottles must have the live yeast renewed each day. It is also most important to have a given number of larvae in each bottle, because tumor incidence is affected by nutrition and the size of the population. Control cultures must be treated bottle for bottle and vial for vial exactly as the experimental cultures, except for the treatment with the compound to be investigated. This means that they must be studied at the same time and place. Control studies done at another time and in another place are not considered comparable. These precautions are necessary because of the variations in tumor incidence within a stock presumably due to uncontrolled environmental conditions.

The method of treatment depends on the chemical tested and the cells which the investigator desires to reach. The solvent is also important. Aerosols enter the body through the spiracles in concentrations which are effective. Chemicals such as nitrogen mustard, when applied in a solvent such as cyclohexane, have been found to act merely when brought in contact with the fly by dropping it on the abdomen. Certain agents are effective when added to the food, while others must be injected into eggs or larvae. In dealing with melanotic tumors it should be realized that they appear quite early in larval life, that the pigmentation visible to the naked eye or under the dissecting microscope appears later, and that treatment should be gauged accordingly. Vaginal douche results in a rather high mortality, but the chromosomes of sperm and eggs may be affected by chemicals introduced by that route.

\* This study was aided by a grant from the National Cancer Institute, Public Health Service.

Received for publication March 16, 1951.

When the contact, aerosol, or vaginal douche methods are used, matings of constant numbers of males and females of the tumor stock are made. One or both parents ( $P_1$ ) are treated, depending on the purpose of the experiment, and the females are allowed to oviposit on food, the surface of which is seeded with yeast. As soon as the larvae hatch, a constant number are transferred to fresh cultures. Usually 200 are placed in each bottle or 8 in each vial. If the larvae ( $P_2$ ) are to be injected, this is done at as early an age as possible, and a constant number is kept in each container. When the chemical is to be ingested, it is contained in the food on which the eggs are laid and to which the larvae ( $P_2$ ) are transferred.

Muller-5 method is that it may be repeated by recovering heterozygous females in the  $F_3$ , in the event that the numbers obtained are insufficient for a conclusive lethal test. The stock may also be retained for subsequent localization of the lethal and study of salivary gland chromosomes. A balanced stock may be made by substituting the  $In(1)AM$  chromosome for the  $sc^S B InS w^a sc^S$  females each generation, since homozygous  $In(1)AM$  females are sterile.

If the chemical is administered in the food, if the larvae are injected, or if only the male is affected by the treatment, the mutation rate is obtained by mating the male in the  $F_2$  generation to virgin Muller-5 females (cross A). The subse-

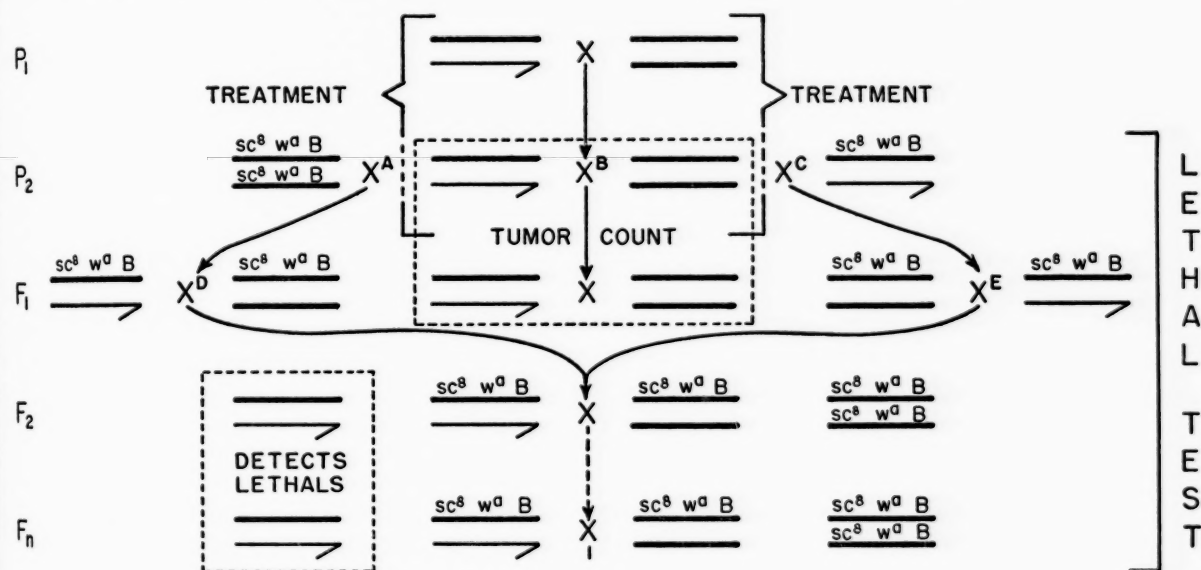


CHART 1

SCHEME FOR DETECTING EFFECT OF TREATMENT ON TUMOR INCIDENCE AND MUTATION RATE

The adults ( $P_2$ ) are carefully examined for the pigmentation denoting the presence of tumors in the larvae. Since they do not cause death of the fly, subsequent generations (cross B) may be obtained for determining mutation rate and incidence of tumors ( $F_1$ ). One advantage of determining tumor incidence in generations subsequent to treatment is that the occurrence of germinal mutation for susceptibility can be ruled out. If the parents have been treated, the lethals will be present in their germ cells and may be detected by mating the virgin female offspring to Muller-5 males (cross C) individually and again mating the  $F_1$  heterozygous females to  $sc^S B InS w^a sc^S$  (Muller-5) males (cross E). The absence of one of the expected classes of males in the  $F_2$  indicates the presence of a lethal on the X chromosome of the missing males. A well known advantage of the

sequent steps are carried out as previously described in  $F_1$ ,  $F_2$ , etc.

Most of the objections to negative results in isolated experiments are equalized if not overcome by this arrangement of crosses, except for the possibility that the agent differs in its ability to affect chromosomes of germ and somatic cells. Mutation rate is tested in germ cells, whereas the hypothetical mutations for initiating tumor formation would occur in somatic cells. Data on visible mutations in mice are also subject to this criticism. There is little doubt that mutation of the proper kind in the germ cells will result in a changed tumor incidence in subsequent generations.

In order to prove that somatic mutations are responsible for the appearance of tumors it would be reasonable to expect that when a chemical

induces an increase in the number of tumors there would be an increase in the rate of mutation (although not necessarily of the same order of magnitude) and that mutagens increase the tumor incidence. If there were no relationship, one would expect to find four classes of chemicals: those which are both carcinogenic and mutagenic, those which are carcinogenic and not mutagenic, those which are mutagenic and not carcinogenic, and those which are neither carcinogenic nor mutagenic. In addition, the method furnishes a means for screening agents which may have mutagenic or carcinogenic action or both. This method has been used in a number of experiments which will be reported in a short series of papers.

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# Tumor Incidence and Lethal Mutation Rate in a Tumor Strain of *Drosophila* Treated with Formaldehyde\*

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The fact that certain agents cause mutations as well as induce tumors is intriguing enough to justify an examination of the possibility that the two processes may be related. Since it has been shown that chemical as well as physical agents can produce mutations, this would add significance to the discovery of any such relationship. Several chemicals have been tested for mutagenic and tumorigenic properties in an attempt to elucidate the relationship between mutation and carcinogenesis (2). It has been possible to design a critical experiment using *Drosophila*, because an objective test for lethal mutation rate can be done at the same time tumor incidence is determined. The results obtained when a tumor-bearing strain of *Drosophila melanogaster* was treated with formaldehyde are reported here. This chemical was chosen because it is known to be a mutagen (1, 4, 5) which is effective only in the male (3), making not only the total incidence but also the sex incidence of tumors valuable when compared to mutation rate.

## METHODS<sup>1</sup>

The strain of *Drosophila* selected for use is the tu 36a strain, discovered by Bridges in 1938, which was obtained through the kindness of Dr. E. W. Hartung. It has a low and fairly constant incidence of spontaneous tumors due to multiple genetic factors. An isogenic stock constructed from this strain has shown no higher incidence of tumors. In view of the known effect of temperature, nutrition, and crowding on tumor incidence, the culture medium was carefully prepared in a uniform manner, and fresh, live yeast was added to the surface each day. The experiment was carried out in an incubator at  $25 \pm 0.5^\circ \text{C.}$ , and parental cultures consisted of a constant number of females and males. Parallel control cultures were also studied.

A preliminary study was made to determine the

\* This project was aided by a grant from the National Cancer Institute, Public Health Service.

<sup>1</sup> The author is indebted to Betty Rosenbohm for technical assistance during the course of this work.

Received for publication February 14, 1951.

most suitable method for the administration and concentration of formaldehyde. In the first group of cultures, 0.75 cc. of a solution of 10 per cent, 15, 20, and 25 per cent formaldehyde were added, respectively, to the surface of 50 cc. of culture medium in each of four bottles on which the fertilized females were allowed to oviposit. In the second group the chemical was thoroughly mixed in the cooling medium immediately before the agar had set so that the final concentration of formaldehyde was 0.10, 0.15, 0.20, and 0.25 per cent, respectively.

After the optimum concentration of formaldehyde had been found, individual matings of parents from the tu 36a strain were made and the sex ratio determined for each pair. Only those flies whose sex ratio was found to be within the usual range were used subsequently. Fifty males and fifty females were mated in bottles partially filled with 50 cc. of laboratory medium containing 0.1 per cent formaldehyde. These parents were removed from the bottles at the end of 24 hours. Five days after hatching, 200 larvae were transferred to a fresh culture bottle containing no formaldehyde. The flies which hatched were examined for tumors, and the males were used for detecting lethal mutations. The tumors were measured by using an ocular micrometer, and a record was made of their location.

When some of the flies were 3 days old, 50 females were transferred to each bottle of culture medium without formaldehyde, and the experiment was repeated for the  $F_1$  generation obtained from that cross. To preclude the possibility that X-chromosome lethals were present before treatment began, a separate series of experiments was performed in vials, with individual matings of the tumor stock. When the larvae were transferred to cultures without formaldehyde, only eight were placed in each vial. Observations were made on the tumor incidence among the flies remaining in the vials and bottles, respectively, after the constant number of larvae had been removed, and also in the  $F_1$  generation obtained from them.

The  $sc^{S1} B InS w^a sc^8$  (Muller-5) stock was used to determine the lethal mutation rate among the

treated and control males. These males were mated individually to virgin, Muller-5 females, and heterozygous female offspring were mated individually to Muller-5 males. The absence of either  $sc^{51} B \text{ InS } w^a sc^8$  or wild type sons from that cross was regarded as evidence for the presence of a lethal on that respective chromosome. When inadequate numbers were obtained it was possible to repeat the test with the heterozygous siblings—one of the advantages of the method. All lethals were retested, and thereby confirmed, at least once in that manner.

### RESULTS

It was not necessary to discard any of the cultures because of atypical sex ratios of the parents ( $P_1$ ). The concentration of formaldehyde which was found to be most suitable was 0.1 per cent (Table 1). It was undesirable to affect the life

TABLE 1

POPULATION AT END OF TENTH DAY FOLLOWING TREATMENT WITH FORMALDEHYDE

Per cent formaldehyde	10	15	20	25	0.10	0.15	0.20	0.25
Larvae	82	21	18			30	48	70
Pupae	55					121	14	2
Adults					316			
Total:	137	21	18		316	151	62	72

TABLE 2

EFFECT OF FORMALDEHYDE ADMINISTRATION ON TUMOR INCIDENCE IN  $P_2$  GENERATION WITH PROVED INCREASE IN LETHAL MUTATION RATE FROM TREATMENT

	Tumors	Population	Per cent tumors	P
♂♂ Treated	7	540	1.30	0.43
♂♂ Without treatment	10	423	2.36	
♀♀ Treated	8	384	2.08	0.31
♀♀ Without treatment	5	438	1.14	
Total treated	15	924	1.62	0.79
Total without treatment	15	861	1.74	

cycle any more than necessary by the treatment, and it is evident that in higher concentrations this chemical was either lethal or delayed eclosion. Kaplan reports a higher percentage of eclosions with the greater concentrations of formaldehyde. Placing the solution on the surface of the food was found to be unsatisfactory, because the parent flies drowned in so much fluid, the growth of yeast on the surface was inhibited, and uniform distribution of the agent was not insured.

There was no detectable effect of treatment on

tumor incidence in the generation ( $P_2$ ) of flies treated with formaldehyde and tested for lethal mutation rate (Table 2). This is true for the entire group and also when the sexes are tabulated separately. In a population of 540 treated males there were 7 with tumors, as compared to 10 among 423 without treatment. There were 8 treated females with tumors among 384, and 5 females

TABLE 3

EFFECT OF FORMALDEHYDE ADMINISTRATION ON TUMOR INCIDENCE IN  $P_2$  GENERATION

	Tumors	Population	Per cent tumors	P
♂♂ Treated	19	1,093	1.74	0.72
♂♂ Without treatment	15	773	2.07	
♀♀ Treated	16	855	1.87	0.96
♀♀ Without treatment	15	789	1.90	
Total treated	35	1,948	1.80	0.85
Total without treatment	30	0,562	1.92	

TABLE 4

EFFECT OF FORMALDEHYDE ADMINISTRATION ON TUMOR INCIDENCE IN  $F_1$  GENERATION WITH PROVED INCREASE IN LETHAL MUTATION RATE FROM TREATMENT

	Tumors	Population	Per cent tumors	P
♂♂ Treated	9	320	2.81	0.64
♂♂ Without treatment	10	448	2.23	
♀♀ Treated	11	272	4.04	0.52
♀♀ Without treatment	14	506	2.77	
Total treated	20	592	3.38	0.36
Total without treatment	24	954	2.52	

without treatment developed tumors out of a population of 438. The  $\chi^2$  test shows that the tumor incidence in treated flies is not significantly different from that in those without treatment ( $P = 0.43$  for the males,  $P = 0.31$  for females, and  $P = 0.79$  for both). In another experiment the tumor incidence was obtained without testing the lethal mutation rate. When these data are added (Table 3), the percentage of tumors following treatment even more closely resembles that among controls, with no indication that the males develop more tumors than the females. A total of 35 tumors was found among 1,948 treated flies and 30 among 1,562 without treatment ( $P = 0.85$ ).

The treatment did not cause an increase in the number of tumors in the following ( $F_1$ ) generation. From the figures in Table 4 it is clear that there is no significant difference in incidence between the

sexes or between those treated and those without treatment. There were 20 tumorous flies among 592 that were treated (3.38 per cent) and 24 among 954 without treatment (2.52 per cent).

Approximately half of the treated larvae were allowed to remain on medium containing formaldehyde until eclosion, and the remainder was removed after 5 days. Neither group had more tumors than the corresponding control group. In the  $P_2$  there were 2.47 per cent tumors (22 out of 889 flies) in those removed after 5 days and 2.12 per cent (18 out of 848 flies) in those without treatment ( $P = 0.71$ ). When the larvae remained on the medium with formaldehyde, there was an incidence of 1.23 per cent (13 among 1,059 flies)

TABLE 5  
SIZE OF TUMORS

	No.	Av. size of tumor (mm. <sup>2</sup> )	Av. length of body (mm.)
♂♂ Treated	28	0.0061	2.33
♂♂ Without treatment	25	0.0092	2.37
♀♀ Treated	27	0.0073	2.79
♀♀ Without treatment	29	0.0083	2.81
Total treated	55	0.0066	2.55
Total without treatment	54	0.0087	2.61

TABLE 6  
EFFECT OF FORMALDEHYDE ADMINISTRATION ON LETHAL MUTATION RATE

	Lethals	Chromosomes tested	Per cent lethals	P
Treated	18	1,174	1.53	0.0001
Control	1	1,217	0.08	

in contrast to 1.68 per cent (12 among 714) in those on ordinary food ( $P = 0.57$ ). The same was true in the  $F_1$  generation. In those flies allowed access to formaldehyde for 5 days there was an incidence of 2.01 per cent (5 among 249 flies) with 3.58 per cent (11 among 307 flies) in the corresponding control cultures ( $P = 0.48$ ). When allowed to remain on the same food until eclosion, there was a tumor incidence of 4.37 per cent (15 among 343) and an incidence of 2.01 per cent (13 among 647 flies) in the group without contact with formaldehyde ( $P = 0.35$ ). Results were no different when vials instead of bottles were used.

No more than one tumor was found in any individual fly. They were invariably located in the abdomen, and a record of their location on an anatomical diagram failed to show any difference in distribution between treated and control groups. Measurement of the tumors revealed a variation of between 0.0001 and 0.073 mm.<sup>2</sup> The average size, which is recorded in Table 5 as the

product of length  $\times$  width, was not greater in those tumors from flies raised in contact with formaldehyde. No relationship between body size and tumor dimensions was found. Although the females were larger than the males, as would be expected, there was no great discrepancy between the average size of tumors in the two sexes.

Quite different results were obtained when the lethal mutation rate on the X chromosome was determined. There were 18 lethals among 1,174 chromosomes treated and tested (1.53 per cent), whereas there was only one lethal among 1,217 chromosomes tested (0.08 per cent) from the control. There is clearly an increase in mutation rate ( $P = 0.0001$ ) following treatment with formaldehyde in 0.1 per cent concentration. All these lethals were retested at least once for confirmation.

## DISCUSSION

Re-examination of mutagenic properties of various chemicals has revealed a number which increase the lethal mutation rate in *Drosophila*. Among them, formaldehyde has been found to be an effective mutagen (1, 3-5) when added to the culture medium. An interesting fact about its action is that only males are affected when it is ingested by the larvae (3). This chemical then should be a suitable one to use in determining the effect of a mutagen not known to be carcinogenic on tumor incidence. Not only should a comparison of tumor incidence be illuminating, but also any difference in incidence between the sexes might be significant, since the mutagen acts only in the male. Examination of the results indicates no change in tumor incidence in spite of an increase in mutation rate following treatment (Tables 2-4). Neither before nor after treatment was there a difference in the incidence of tumors between the sexes. There is, therefore, no evidence that the chemical exerts a direct tumorigenic action or one mediated through an increased mutation rate.

To minimize the possibility that the chemical might be effective not because of direct action but because of action on the parent of the animal in which a tumor appeared, counts were made for tumors in both  $P_2$  and  $F_1$  generations. In neither instance was a difference between control and experimental cultures found. There was a higher average incidence of tumors in the  $F_1$  than in the  $P_2$  generation, but the difference is not statistically significant. It is believed that this is merely a reflection of the usual variation in number of tumors when one group of cultures is compared to another raised at different times. Great care was taken throughout the experiment to have the un-

treated cultures as nearly comparable as possible to those which were treated in time, temperature, location, and handling. Without this precaution results would be valueless, because the percentage of tumors appearing may fluctuate under ordinary laboratory conditions from one generation to the next. The importance of determining mutation rate at the same time that tumor incidence is computed should likewise be emphasized.

Although testing carcinogens for mutagenic activity is the usual procedure for investigating the relationship between the two, one perhaps should equally expect that substances increasing mutation rate should increase tumor incidence if somatic mutation is responsible for the appearance of tumors—particularly since no chemical or physical agents are known to cause specific mutations. In another study (2), no increase in mutation rate following treatment with 20-methylcholanthrene was found. With the lethal mutation rate as an objective index, the results obtained in this study indicate that an increase in mutation rate did not increase the tumor incidence.

Since there is a difference in lethal mutation rate between males and females, the question of a possible difference between mutation rate of lethals and mutation rate of tumor genes may arise. Present evidence indicates, however, that when chemicals alter mutation rate they do not act selectively on certain genes. Although improbable, the possibility that the chemical is not reaching the chromosomes in somatic cells, even though germinal chromosomes are affected, is not eliminated in the experiment.

The failure to find any change in tumor incidence is more significant than in an isolated experiment, since an increase in mutation rate occurred at the same time, proving that the chemical was raising the rate of mutation in certain cells. The results, therefore, are not regarded as negative in the usual sense of the term. It is possible that the increase in lethal mutation rate is detected because there are more loci on the X chromosome capable of such a change, while mutations of loci affecting tumorigenesis on the X chromosome were not detected because of their

smaller number. It is equally true, however, that the chance of detection depends not only on the number of loci but also on the number of cells tested. The lethals were detected in a specific number of X chromosomes, whereas the genes affecting tumor incidence in numerous somatic cells in each of all the flies examined may have been affected by treatment. In addition, mutation of tumor genes on the remainder of the chromosomes may also be detected.

In view of the unchanging incidence of tumors in both males and females, in the presence of an increased lethal mutation rate after treatment with a chemical known to increase lethal mutation rate only in the male, the least that can be said is that these results do not support the somatic mutation theory of the etiology of tumors.

### CONCLUSIONS

1. The lethal mutation rate on the X chromosome was increased in males tested after treatment with 0.1 per cent formaldehyde.
2. Determination of tumor incidence at the same time indicated that there was no significant change in the percentage of tumors in the same or the following generation.
3. Although it is known that formaldehyde increases the lethal mutation rate only in the male, incidence of tumors in both males and females was not significantly different from that of comparable groups without treatment.
4. The results do not support the hypothesis that atypical growth is the result of somatic mutation.

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# The Effect of Visible Light on the Development in Mice of Skin Tumors and Leukemia Induced by Carcinogens\*

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The absence of light enhances epidermal carcinogenesis in Swiss albino (9) and C57 black (10) strains of mice painted with a solution of 3,4-benzpyrene. It now appears that the presence or absence of light affects the response of mice of the dilute brown (DBA) strain to percutaneous administration of either 3,4-benzpyrene or 20-methylcholanthrene by altering the incidence of not only skin carcinomas but leukemias as well. These mice are peculiarly susceptible to the leukemogenic action of several carcinogenic hydrocarbons, although the incidence of spontaneous leukemia in the strain is low (2, 4-6, 11).

## EXPERIMENTAL PROCEDURE

Mice of the dilute brown strain obtained from the Biological Station of the Roswell Park Memorial Institute, through the courtesy of Dr. W. S. Murray and Dr. S. G. Warner, were divided into groups of 50. The sexes were equally distributed among the groups but were segregated to prevent breeding. The mice were housed in wire mesh cages in specially constructed rooms, the details of which have been described (3). No light was admitted to the dark room, while the light room was illuminated for 12 hours each day by fluorescent lamps, as in the experiments reported earlier (9, 10). The diet consisted of unlimited quantities of Purina Dog Chow and water. The mice were 6-7 weeks old at the first painting.

The first experiment was conducted between December, 1941, and July, 1942. Forty mice from each group were painted twice weekly with a 0.5 per cent solution of 3,4-benzpyrene in benzene, applied to the interscapular region with a No. 8 camel's hair brush. Thirty-five such paintings were given over a period of 17 weeks. The mice were observed for an additional 60 days after cessation of the applications. Ten untreated mice of each group served as controls.

The second experiment, conducted between

\* Aided by a grant from the Rockefeller Foundation and the Jane Coffin Childs Memorial Fund for Medical Research.

Received for publication April 7, 1951.

January and July, 1949, was performed with 40 treated mice and 10 untreated controls in each group. Litter-mates were distributed equally between those in the light and dark rooms. Each of the treated mice was painted twice weekly with a 0.5 per cent solution of 20-methylcholanthrene in benzene. The solution was applied to nine successive sites in rotation as previously described (6). Paintings were continued until most of the mice had developed either leukemia or epidermoid carcinomas.

All animals were allowed to die or were sacrificed when they appeared moribund. Autopsies were performed and appropriate specimens were examined histologically.

The experimental design provided a relatively potent stimulus for epidermal carcinogenesis in the first experiment and a relatively strong leukemogenic stimulus in the second experiment.

## RESULTS

One mouse died from no ascertainable cause in each of the experimental groups before the first neoplasm appeared in any member of the group. Each group, then, contained 39 treated mice. The untreated control mice were sacrificed at termination of the experiments. None of them developed a neoplasm.

Topical application of 3,4-benzpyrene solution to the mice in the first experiment produced epilation of the interscapular region, followed by growth of hair, particularly among those mice kept in the dark. No difference in hair growth was observed after the nineteenth painting, but all the mice in the light room always seemed more excitable and difficult to handle. Dermatitis comparable to that found in C57 black mice painted with a benzpyrene solution (10) was not observed.

Mortality curves for the mice in the first experiment are depicted in Chart 1, and the incidence of epidermoid carcinoma and leukemias is presented in Table 1. Some mice had both lesions. The difference in incidence of the two anatomical types of neoplasia between the light and dark room mice is

statistically significant, for  $P$  is far less than 0.01 when the data are analyzed by the  $\chi^2$  method. The more rapid mortality among the mice housed in the light room probably reflects the highly lethal

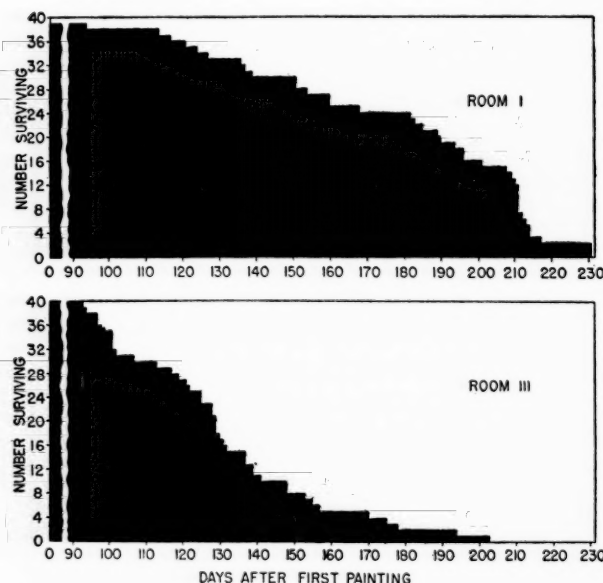


CHART 1.—Mortality curves for mice kept in dark (Room I) and light (Room 3) and painted with 0.5 per cent solution of 3,4-benzpyrene in benzene.

TABLE 1  
TUMOR INCIDENCE  
(3,4-Benzpyrene)

	Total No.	Epidermoid Carcinoma		Leukemia	
		No.	Per cent	No.	Per cent
Dark	39	27	69	13	33
Light	39	11	28	31	79

characteristics of hydrocarbon-induced leukemia in mice as compared to the more chronic course of epidermoid carcinoma.

The mortality curves for the second experiment (Chart 2) again reveal that the mice exposed to light died more rapidly than did those kept in the dark room. The incidence of leukemias and epidermoid cancers is listed in Table 2. Differences in the distribution of leukemias between the two groups are significant at a level of 0.03. The difference in distribution of epidermoid carcinomas is not statistically significant. The higher incidence of leukemia (Table 2) in this experiment produces a marked decrease in survival time for both groups as contrasted with the mice in the first experiment. Hyperexcitability of the mice exposed to light was readily apparent, but no difference in epilation could be noted in the two groups.

The leukemias observed were identical in all

respects with the mediastinal lymphoma and general lymphomatosis that follow percutaneous application of carcinogenic hydrocarbons in mice of the dilute brown strain, as reported previously (6). The disseminated type of disease was found far more commonly than the localized mediastinal lesion. The diagnosis of epidermoid carcinoma required that neoplastic cells infiltrate the pancreaticus carnosus.

## DISCUSSION

Epidermal carcinogenesis by 3,4-benzpyrene is strongly influenced by the presence or absence of light in at least three different strains of mice. Ultraviolet irradiations cannot be incriminated in this reaction, since they were not present in the radiations used in our experiments. Doniach and

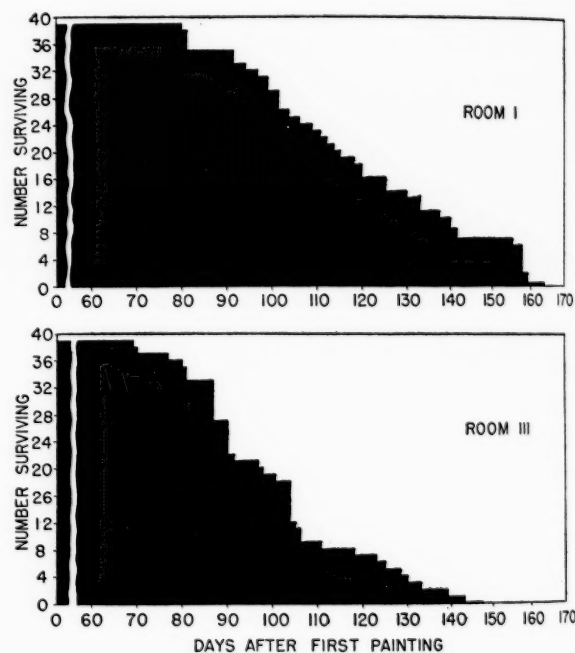


CHART 2.—Mortality curves for mice kept in dark (Room I) and light (Room 3) and painted with 0.5 per cent solution of 20-methylcholanthrene in benzene.

TABLE 2  
TUMOR INCIDENCE  
(20-Methylcholanthrene)

	Total No.	Epidermoid Carcinoma		Leukemia	
		No.	Per cent	No.	Per cent
Dark	39	9	23	29	74
Light	39	3	8	36	92

Mottram (1), however, showed that fewer skin cancers occurred among mice treated with 3,4-benzpyrene and exposed to direct sunlight than among those which received no ultraviolet irradiation. They thought that strong sunlight reduced

tumor production by increasing dermatitis caused by the photodynamic properties of the carcinogen. Light produced no significant dermatitis in two of the three strains of mice studied in our experiments, but the effect on epidermal carcinogenesis was similar in all. Therefore, dermatitis does not appear to be a major factor in producing the different responses to 3,4-benzpyrene in the light and in the dark.

The nutritional state of the subject may affect its response to a carcinogenic agent. Failure to demonstrate any difference in weight between the mice kept in the light and those in the dark rooms eliminates pronounced difference in nutritional status as a major influence in eliciting the different reactions observed.

Strong circumstantial evidence implicates the formation of protein-carcinogen complexes as an important factor in the pathogenesis of hepatic tumors produced by azo dyes (8). An analogous situation is suggested in the production of carcinoma of the skin. E. C. Miller (7) found that the application of 3,4-benzpyrene to the skin of mice produced protein-bound fluorescent substances only in the epidermis. Exposure of the mice to sunlight or to light from incandescent bulbs reduced the levels of epidermal protein-bound derivatives significantly, as compared to the concentrations attained by mice kept in the dark. The direction of the change parallels the light effect on epidermal carcinogenesis.

Miller suggested that the diminished levels of 3,4-benzpyrene derivatives and the lower skin tumor incidence in the presence of light might result from partial photo-oxidation of the carcinogen, thereby decreasing the effective dose of the agent. If this is the case, metabolites of 3,4-benzpyrene or 20-methylcholanthrene, rather than the parent substances themselves, must be leukemogenic. Weigert, Calcutt, and Powell have discovered only one metabolite of 3,4-benzpyrene in painted mouse skin, but its chemical nature and biologic properties have not been established (12). Other metabolites are known to be formed during passage of the carcinogen through the body (13). The reciprocal relationship between the incidence of skin cancer and leukemia in our experiments might be interpreted to suggest that light facilitates absorption through the skin of unchanged carcinogen or a leukemogenic derivative, permitting it to act upon the lymphoid tissue.

The second experiment, using 20-methylcholanthrene, provides a relatively great stimulus for leukemogenesis. The reciprocal relationship between skin cancer and leukemia production in the light and dark is similar to that observed among

the mice treated with 3,4-benzpyrene. The reduction in statistical significance of the differences observed agrees with the general principle that physiologic influences in tumor production may be masked by overwhelming carcinogenic stimuli.

A full explanation of the effects of the radiations used on tumorigenesis must await further experiment.

#### SUMMARY

Mice of the dilute brown (DBA) strain kept in an environment of visible light for 12 hours daily or in complete darkness were painted with solutions of 3,4-benzpyrene or 20-methylcholanthrene. The incidence of leukemia among the mice exposed to light was significantly higher than in those kept in the dark. Fewer animals exposed to light developed skin carcinomas.

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# Comments and Communications

## COMMENTS ON A RECENT REVIEW

BY W. C. SCHNEIDER AND G. H. HOGEBOOM

ON

## THE ISOLATION OF CELL COMPONENTS BY DIFFERENTIAL CENTRIFUGATION

In a recent review article on the isolation of cell components by differential centrifugation (8), Schneider and Hogeboom make certain statements concerning the isolation and composition of cell nuclei about which I should like to comment.

1. The idea is advanced as it has been elsewhere (5) that for results of enzyme studies on isolated particulates to be of significance, a large fraction of the total amount of cellular enzyme in question should be found in the particulate in question. This reasoning should not be generally applied to cell nuclei, since the latter may comprise only a small fraction of the cell volume. Furthermore, it seems unprecedented in chemical or biochemical research to insist (as the reviewers appear to) that yield rather than purity is the important factor in obtaining material fit for chemical analysis.

2. On the basis of relative concentrations, the reviewers appear to be inconsistent in refusing to assign significance to the presence of a number of enzymes in isolated cell nuclei while they do assign probable significance to the presence of PNA in isolated mitochondria.

3. The arbitrary general statement of the reviewers that in their experience the Waring Blendor is unsatisfactory for preparing cell homogenates suitable for cell fractionation studies is contrary to the experience of workers in this and many other laboratories. The authors ignore the effect of the medium employed and the fact that the speed at which the blendor is run can be controlled over a wide range by the use of a rheostat.

4. The reviewers note that many of the nuclei prepared in very dilute citric acid at pH 6.0 have lost their spherical shape and optical homogeneity, and seem to imply that sphericity and optical homogeneity are necessary criteria for good nuclei. However, the use of isotonic sucrose rather than hypertonic sucrose is now advocated by the authors in isolating mitochondria, although the morphology of the mitochondria is thereby altered.

It was stated some time ago that repeated washing of unbroken liver cells with isotonic saline solution could remove over 50 per cent of the protein of these cells without altering their microscopic appearance (6). Hence, microscopic appearance is not proof of biochemical integrity of a cell, or for that matter of any cell particulate.

Condensation of the chromatin occurs when cell nuclei are isolated at pH 6.0, presumably as the result of simple dehydration. A similar condensation of the chro-

matin of chromosomes isolated in physiological saline can be reversed by the use of sucrose (7). Chromatin condensation indicates neither protein denaturation nor enzyme inactivation when brought about by mild means.

Electron microscope photographs of nuclear membranes (2), as well as the swelling behavior of nuclei in sucrose solutions, indicate that the nuclear membrane is more permeable than the mitochondrial membrane and should not at present be relied upon to prevent loss of material from the nuclei or gain of material by them.

5. The reviewers claim that my work on cell nuclei is contrary to the results of K. Arnesen, Y. Goldsmith, and A. D. Dulaney (1), who stated that some nucleic acid is lost from cell nuclei isolated in sucrose solutions unless the pH is lowered to 6.0. As far as I am able to see, my work has no bearing whatsoever on this point and should not have been cited in this connection.

6. In quoting my analyses of nuclei prepared at pH 6.0 and pH 4.0 for total lipid (3), the reviewers have compared the highest value listed for nuclei prepared at pH 6.0 with the two lowest values listed for nuclei prepared at pH 4.0. Ignoring a possible strain difference, the average for all nuclei prepared at pH 6.0 is 9.7 per cent, and the average for all nuclei prepared at pH 4.0 is 5.3 per cent, instead of the values reported by the reviewers (10.8 for nuclei prepared at pH 6.0 and 3.2 for nuclei prepared at pH 4.0).

7. The reviewers state that our work indicates the absence of phospholipid in cell nuclei, but they have not mentioned that some of the data referred to were derived from studies of defatted nuclei. Moreover, the reviewers have not mentioned the papers of Haven and Levy (4) and of Williams and collaborators (9), showing the presence of phospholipid in cell nuclei by direct analysis, and have not made it clear that it is their conclusion, not ours, that cell nuclei do not contain phospholipid.

Owing to lack of space, it is only possible to point out the above-mentioned disagreements with the reviewers without presenting complete arguments in rebuttal.

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## Book Reviews

*Cell Physiology and Pharmacology.* By J. F. DANIELLI.  
New York: Elsevier Publishing Co., 1950. Pp. 156.  
\$3.00.

This slender volume, based upon a series of lectures given at University College in London, will appeal to a wide audience: the biochemistry student in quest of stimulating supplementary reading and the pharmacology lecturer in search of new pedagogic approaches, the practicing cell physiologist with a desire for theoretical considerations and the theoretician in need of illustrative examples, the biologist with a guilty conscience about physical chemistry and the chemist with a vague awareness of the horizons of biology.

A random sampling of the subject index will give an idea of the scope of the material which is presented: adrenaline, ameboid movement, BAL, cancer, curare, dielectric constant of cellular systems, enzyme poisons, genes, hexokinase, ions, lysis, mastitis in cattle, mitosis, nerve, osmic acid, partition coefficients, pyridoxal, renal secretion, surface tension, trypanosomes, urethan, viruses, xanthopterin. This breadth of vision is the strength of the book; its weakness is exemplified by the fact that, of the more than 200 entries in the subject index, less than two dozen receive more than cursory mention. Many readers will be a little disappointed that Dr. Danielli did not expand this short series of lectures into a full 1-year course.

The volume is formally divided into six sections entitled: "The Cell as a Physico-chemical Unit"; "Possible Actions of Drugs on Surfaces"; "Membrane Permeability and Drug Action"; "Enzymes and Drug Action"; "The Actions of Narcotics"; and "Responses of Cells on the Biological Level." In model lecture-hall fashion, the discussion passes from general considerations to specific illustrations (reproduced here in 21 figures, 22 tables, and 3 plates), to correlations with previously presented material, and finally to those aspects of the problem which might most profitably be investigated in the future. Over 100 references to other books, reviews, and key papers in the literature provide an introduction to further reading.

The technical aspects of the volume are most satisfactory: the type is readable and restful to the eye, the figures are reproduced with clarity and accuracy, the paper is of good quality. The liberal use of subheadings was particularly welcome to this reviewer. Dr. Danielli's book will find a place on many bookshelves, right between the standard texts and the daily research notebooks.

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